

A P P L I C A T I O N

for

UNITED STATES LETTERS PATENT

on

Human N-Methyl-D-Aspartate Receptor Subunits,  
Nucleic Acids Encoding Same and Uses Therefor

by

Lorrie P. Daggett,  
Steven B. Ellis,  
Chen Wang Liaw, and  
Chin-Chun Lu

Number of Drawings: Six

Docket No.: P41 9424

Attorneys

Pretty, Schroeder, Brueggemann & Clark  
444 South Flower Street, Suite 2000  
Los Angeles, California 90071

204070 4663007

Human N-Methyl-D-Aspartate Receptor Subunits,  
Nucleic Acids Encoding Same and Uses Therefor

This application is a continuation-in-part of United States Serial No. 08/052,449, filed April 20, 1993, now pending.

204070-2E68E007  
5 The present invention relates to nucleic acids and receptor proteins encoded thereby. Invention nucleic acids encode novel human N-methyl-D-aspartate (NMDA) receptor subunits. The invention also relates to methods for making such receptor subunits and for using the receptor proteins in assays designed to identify and  
10 characterize compounds which affect the function of such receptors, e.g., agonists and antagonists of NMDA receptors.

BACKGROUND OF THE INVENTION

15 The amino acid L-glutamate is a major excitatory neurotransmitter in the mammalian central nervous system. Anatomical, biochemical and electrophysiological analyses suggest that glutamatergic systems are involved in a broad array of neuronal processes, including fast excitatory synaptic transmission, regulation of neurotransmitter  
20 releases, long-term potentiation, learning and memory, developmental synaptic plasticity, hypoxic-ischemic damage and neuronal cell death, epileptiform seizures, as well as the pathogenesis of several neurodegenerative disorders. See generally, Monaghan et al., Ann. Rev. Pharmacol.  
25 Toxicol. 29:365-402 (1980). This extensive repertoire of functions, especially those related to learning, neurotoxicity and neuropathology, has stimulated recent attempts to describe and define the mechanisms through which glutamate exerts its effects.

30 Currently, glutamate receptor classification schemes are based on pharmacological criteria. Glutamate

has been observed to mediate its effects through receptors that have been categorized into two main groups: ionotropic and metabotropic. Ionotropic glutamate receptors contain integral cation-specific, ligand-gated ion channels, whereas metabotropic glutamate receptors are G-protein-coupled receptors that transduce extracellular signals via activation of intracellular second messenger systems. Ionotropic receptors are further divided into at least two categories based on the pharmacological and functional properties of the receptors. The two main types of ionotropic receptors are N-methyl-D-aspartic acid (NMDA) and kainic acid (KA)/ $\alpha$ -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), formerly called the quisqualic acid, or QUIS, receptor. While the metabotropic receptors bind to some of the same ligands that bind to ionotropic glutamate receptors, the metabotropic receptors alter synaptic physiology via GTP-binding proteins and second messengers such as cyclic AMP, cyclic GMP, diacylglycerol, inositol 1,4,5-triphosphate and calcium [Gundersen et al., Proc. R. Soc. London Ser. 221:127 (1984); Sladeczek et al., Nature 317:717 (1985); Nicoletti et al., J. Neurosci. 6:1905 (1986); Sugiyama et al., Nature 325:531 (1987)].

The electrophysiological and pharmacological properties of the glutamate receptors have been studied using animal tissues and cell lines, as well as recombinantly produced non-human receptors, as the source of such receptors. The value of such studies for application to the development of human therapeutics has been limited by the availability of only non-human receptor subunits. Moreover, it is only recently that the characteristics and structure of glutamate receptors have been investigated at the molecular level. The majority of such investigation has, however, been carried out in non-human species. Because of the potential physiological and pathological significance of glutamate receptors, it would be desirable (for example, for drug screening assays) to

have available human sequences (i.e., DNA, RNA, proteins) which encode representative members of the various glutamate receptor subtypes. The availability of such human sequences will also enable the investigation of  
5 receptor distribution in humans, the correlation of specific receptor modification with the occurrence of various disease states, etc.

#### BRIEF DESCRIPTION OF THE INVENTION

The present invention discloses novel nucleic  
10 acids encoding NMDA receptor protein subunits and the proteins encoded thereby. In a particular embodiment the novel nucleic acids encode NMDAR1 and NMDAR2 subunits of human NMDA receptors. More specifically, the invention  
15 nucleic acids encode NMDAR1, NMDAR2A, NMDAR2B, NMDAR2C and NMDAR2D subunits that contribute to the formation of NMDA-activated cation-selective ion channels. In addition to being useful for the production of NMDA receptor subunit  
20 proteins, these nucleic acids are also useful as probes, thus enabling those skilled in the art, without undue experimentation, to identify and isolate nucleic acids encoding related receptor subunits.

Functional glutamate receptors can be assembled, in accordance with the present invention, from a plurality of NMDA receptor subunit proteins of one type (homomeric)  
25 or from combinations of subunit proteins of different types (heteromeric).

In addition to disclosing novel NMDA receptor protein subunits, the present invention also comprises methods for using such receptor subunits to identify and  
30 characterize compounds which affect the function of such receptors, e.g., agonists, antagonists, and modulators of glutamate receptor function. The invention also comprises

204079 ZESB00T  
10038537 040402

methods for determining whether unknown protein(s) are functional as NMDA receptor subunits.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of various human NMDAR1 clones of the invention, with partial restriction maps of each clone. The clones are aligned and the differences in the DNAs (i.e., deletions and insertions), relative to clone NMDA10, are indicated. Translation initiation and termination sites are represented by a "V" and a "\*", respectively. Insertions are marked as inverted triangles, deletions are indicated by spaces in the boxes. The numbers above the insertions and deletions refer to the number of nucleotides inserted or deleted relative to NMDA10.

Figure 2 is a schematic representation of cDNAs encoding full-length human NMDAR1 subunit subtypes of the invention, with partial restriction maps of each DNA. The full-length cDNAs are constructed by ligation of appropriate portions of the clones shown in Figure 1. Regions of each full-length cDNA composed of nucleotide sequences corresponding to a particular clone are distinguished as solid, striped, cross-hatched or open boxes.

Figure 3 presents the entire nucleotide sequence of construct NMDAR1A (see Sequence ID No. 1) with the following information added for ease of comparison of the splice variations of the NMDAR1 subunit transcript: lowercase letters indicate 5' untranslated sequence and the 3' untranslated sequence of the NMDAR1 splice variant shown in Sequence ID No. 1 (in some of the other splice variants, this 3' untranslated sequence is actually coding sequence); uppercase letters indicate coding sequence; the translation initiation codon is identified by the word "START" whereas

the three different translation termination codons (TGA) used in the different splice variants are identified by small boxes; significant restriction enzyme sites used in preparing full-length variant constructs are identified by name above the sites; the location of a 63-bp insertion (see Sequence ID No. 3) that exists in some of the variants is marked as "63 bp INSERT"; the nucleotide sequences that are deleted from some of the variants are boxed and labeled as "204 bp DELETION," "363 bp DELETION," and "1087 bp DELETION."

Figure 4 is a schematic representation of various human NMDAR2C clones of the invention, with partial restriction maps of each clone. The clones are aligned and the differences in the DNAs relative to clone NMDA26 are indicated in the same manner as done in Figure 1.

Figure 5 is a schematic representation of full-length human NMDAR2C subunit subtypes of the invention, with partial restriction maps of each DNA. The full-length cDNAs are constructed by ligation of appropriate portions of the clones shown in Figure 4. Regions of each full-length cDNA composed of nucleotide sequences corresponding to a particular clone are distinguished as solid, striped, cross-hatched or open boxes.

Figure 6 presents restriction maps of CMV promoter-based vectors pCMV-T7-2 and pCMV-T7-3.

#### DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided isolated nucleic acids encoding human N-methyl-D-aspartate (NMDA) receptor subunit(s). In one aspect of the present invention, nucleic acids encoding NMDA receptor subunit(s) of the NMDAR1 subtype are provided. In another aspect, nucleic acids encoding NMDA

receptor subunit(s) of the NMDAR2 subtype are provided. In a further aspect, eukaryotic cells containing such nucleic acids, and eukaryotic cells expressing such nucleic acids are provided.

5 Also provided are protein(s) encoded by the above-described nucleic acids, as well as antibodies generated against the protein(s). In other aspects of the present invention, there are provided nucleic acid probes comprising at least NMDA receptor subunit-selective  
10 portions of the above-described nucleic acids.

As employed herein, the phrase "human N-methyl-D-aspartate (NMDA) receptor subunit(s)" refers to recombinantly produced (i.e., isolated or substantially pure) proteins which participate in the formation of a  
15 voltage-sensitive cation-selective channel activated by exposure to NMDA, and having at least one transmembrane domain, a large N-terminal extracellular domain, and the like, including variants thereof encoded by mRNA generated by alternative splicing of a primary transcript, and  
20 further including fragments thereof which retain one or more of the above properties.

Use of the phrase "recombinantly produced", "isolated" or "substantially pure" in the present specification and claims as a modifier of DNA, RNA,  
25 polypeptides or proteins means that the DNA, RNA, polypeptides or proteins so designated have been produced in such form by the hand of man, and thus are separated from their native *in vivo* cellular environment. As a result of this human intervention, the recombinant DNAs,  
30 RNAs, polypeptides and proteins of the invention are useful in ways that the DNAs, RNAs, polypeptides or proteins as they naturally occur are not, such as identification of selective drugs or compounds.

The term "functional", when used herein as a modifier of receptor protein(s) of the present invention, means that binding of NMDA (or NMDA-like) ligand to receptors comprising the protein(s) causes the receptor  
5 "ion channels" to open. This allows cations, particularly  $\text{Ca}^{2+}$ , as well as  $\text{Na}^{+}$  and  $\text{K}^{+}$ , to move across the membrane. Stated another way, "functional" means that a signal is generated as a consequence of agonist activation of receptor protein(s).

10 As used herein, a splice variant refers to variant NMDA receptor subunit-encoding nucleic acid(s) produced by differential processing of primary transcript(s) of genomic DNA, resulting in the production of more than one type of mRNA. cDNA derived from  
15 differentially processed primary transcript will encode NMDA receptor subunits that have regions of complete amino acid identity and regions having different amino acid sequences. Thus, the same genomic sequence can lead to the production of multiple, related mRNAs and proteins. Both  
20 the resulting mRNAs and proteins are referred to herein as "splice variants".

Accordingly, also contemplated within the scope of the present invention are DNAs that encode NMDA receptor subunits as defined above, but that by virtue of degeneracy  
25 of the genetic code do not necessarily hybridize to the disclosed DNA under specified hybridization conditions. Such subunits also contribute to the formation of functional receptor, as assessed by methods described herein or known to those of skill in the art, with one or  
30 more additional NMDA receptor subunits of the same or different type (the presence of additional subunits of a different type is optional when said subunit is an NMDAR1 subunit). Typically, unless an NMDA receptor subunit is encoded by RNA that arises from alternative splicing (i.e.,  
35 a splice variant), NMDA receptor subunit-encoding DNA and

20409072682001



the NMDA receptor subunit encoded thereby share substantial sequence homology with at least one of the NMDA receptor subunit DNAs (and proteins encoded thereby) described herein. It is understood that DNA or RNA encoding a splice variant may share less than 90% overall sequence homology with the DNA or RNA provided herein, but include regions of nearly 100% homology to a DNA fragment described herein, and encode an open reading frame that includes start and stop codons and encodes a functional NMDA receptor subunit.

10

As employed herein, the phrase "NMDA receptor subunit(s) of the NMDAR1 subtype" refers to proteins which, by hydrophobicity analysis of deduced amino acid sequences, are believed to contain four or more putative transmembrane domains, preceded by a large extracellular N-terminal domain. The amino acid sequence typically contains possible phosphorylation sites for  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase type II and protein kinase C [see, for example, Kemp et al. (1990) Trends in Biological Science Vol. 15:342-346; Kishimoto et al. (1985) J. Biol. Chem. Vol. 260:12492-12499; Whittemore et al. (1993) Nature 364:70-73]. (These protein kinases reportedly play a crucial role in induction and maintenance of long term potentiation.)

25

The putative TMII segment (i.e., second transmembrane domain) is typically flanked by a glutamic acid residue at the extracellular side and a stretch of glutamic acid residues at the cytoplasmic side. This segment contains an asparagine residue believed to be responsible for high  $\text{Ca}^{2+}$  permeability of the NMDAR channel.

30

For a summary of NMDAR properties, see Ben-Ari et al., in TINS 15:333-339 (1992), especially at p. 334.

Exemplary DNA sequences encoding human NMDAR1 subunits are represented by nucleotides which encode substantially the same amino acid sequence as set forth in

35

10038937.010402

Sequence ID Nos. 2, 2E, 2F, 2G, 2H, 2I, 2J, 2K, 2L, 2M, 2N, or 2P. Presently preferred sequences encode substantially the same amino acid sequence as set forth in Sequence ID Nos. 2, 2E, 2F, 2G, 2H, 2I or 2P.

5 Exemplary DNA can alternatively be characterized as those nucleotide sequences which encode a human NMDAR1 subunit and hybridize under high stringency conditions to substantially the entire sequence of any one of Sequence ID Nos. 1, 1A, 1B, 1C, 1D, 1E, 1F, 1G, 1H, 1I, 1J, 1K, 1L, 1M,  
10 1N, or 1P, or substantial portions thereof (i.e., typically at least 25-30 nucleotides thereof); preferably exemplary DNA will hybridize under high stringency conditions to substantially the entire sequence of any one of Sequence ID Nos. 1, 1E, 1F, 1G, 1H, 1I or 1P, or substantial portions  
15 thereof.

Stringency of hybridization is used herein to refer to conditions under which polynucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting  
20 temperature ( $T_m$ ) of the hybrids.  $T_m$  can be approximated by the formula:

$$81.5^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G+C) - 600/l,$$

where  $l$  is the length of the hybrids in nucleotides.  $T_m$  decreases approximately 1-1.5°C with every 1% decrease in  
25 sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridization reaction is performed under conditions of lower stringency, followed by washes of varying, but higher, stringency. Reference to  
30 hybridization stringency relates to such washing conditions. Thus, as used herein:

- 5 (1) HIGH STRINGENCY conditions, with respect to fragment hybridization, refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C (i.e., if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C;
- 10
- 15 (2) MODERATE STRINGENCY conditions, with respect to fragment hybridization, refers to conditions equivalent to hybridization in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C;
- 20 (3) LOW STRINGENCY conditions, with respect to fragment hybridization, refers to conditions equivalent to hybridization in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 42°C, followed by washing in 1X SSPE, 0.2% SDS, at 50°C; and
- 25
- 30 (4) HIGH STRINGENCY conditions, with respect to oligonucleotide (i.e., synthetic DNA  $\leq$  about 30 nucleotides in length) hybridization, refers to conditions equivalent to hybridization in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 42°C, followed by washing in 1X SSPE, and 0.2% SDS at 50°C.

It is understood that these conditions may be duplicated using a variety of buffers and temperatures and that they are not necessarily precise.

Denhart's solution and SSPE (see, e.g., Sambrook, Fritsch, and Maniatis, in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989) are well known to those of skill in the art as are other suitable hybridization buffers. For example, SSPE is pH 7.4 phosphate-buffered 0.18M NaCl. SSPE can be prepared, for example, as a 20X stock solution by dissolving 175.3 g of NaCl, 27.6 g of  $\text{NaH}_2\text{PO}_4$  and 7.4 g EDTA in 800 ml of water, adjusting the pH to 7.4, and then adding water to 1 liter. Denhart's solution (see, Denhart (1966) Biochem. Biophys. Res. Commun. 23:641) can be prepared, for example, as a 50X stock solution by mixing 5 g Ficoll (Type 400, Pharmacia LKB Biotechnology, INC., Piscataway, NJ), 5 g of polyvinylpyrrolidone, 5 g bovine serum albumin (Fraction V; Sigma, St. Louis, MO) water to 500 ml and filtering to remove particulate matter.

Especially preferred sequences are those which have substantially the same nucleotide sequence as the coding sequences in any one of Sequence ID Nos. 1, 1E, 1F, 1G, 1H, 1I, 1J, 1K, 1L, 1M, 1N, or 1P; with those having substantially the same sequence as the coding sequence in Sequence ID Nos. 1, 1E, 1F, 1G, 1H, 1I or 1P being most preferred.

As used herein, the phrase "substantial sequence homology" refers to nucleotide sequences which share at least about 90% identity, and amino acid sequences which typically share more than 95% amino acid identity (>99% amino acid identity when dealing with NMDAR1 subunits). It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology arising as splice variants or

that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

As used herein, the phrase "substantially the same" refers to the nucleotide sequences of DNA, the ribonucleotide sequences of RNA, or the amino acid sequences of protein, that have slight and non-consequential sequence variations from the actual sequences disclosed herein. Species that are "substantially the same" are considered to be equivalent to the disclosed sequences, and as such are within the scope of the appended claims. In this regard, "slight and non-consequential sequence variations" mean that sequences that are substantially the same as the DNA, RNA, or proteins disclosed and claimed herein, are functionally equivalent to the human-derived sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions as the human-derived nucleic acid and amino acid compositions disclosed and claimed herein. In particular, functionally equivalent DNAs encode human-derived proteins that are the same as those disclosed herein or that have conservative amino acid variations, such as substitution of a non-polar residue for another non-polar residue or a charged residue for a similarly charged residue. These changes include those recognized by those of skill in the art as those that do not substantially alter the tertiary structure of the protein.

As employed herein, the phrase "NMDA receptor subunit(s) of the NMDAR2 subtype" refers to proteins which have a large putative extracellular domain at the amino-terminal region. Otherwise, the deduced structure of NMDAR2 subunits displays the same general characteristics as the NMDAR1 subunit structure. A notable typical exception is that the negatively charged glutamic acid

residues that are generally present in the putative TMII segment of NMDAR1 subunits are generally absent from the TMII segment of NMDAR2. Instead, NMDAR2 subunits may contain a positively charged lysine residue in TMII.

- 5 Unlike NMDAR1 subunits, NMDAR2 subunits generally do not form homomeric NMDA receptors. Moreover, the amino acid sequences of NMDAR1 and NMDAR2 subunits are generally less than 50% identical, with identities of less than 30% typically observed.

- 10 NMDAR2 subunits contemplated by the present invention include NMDAR2A, NMDAR2B, NMDAR2C and NMDAR2D types of subunits. Exemplary DNA sequences encoding human NMDAR2A subunits, or portions thereof, are represented by nucleotides which encode substantially the same amino acid  
15 sequence as set forth in Sequence ID No. 11, or substantially the same amino acid sequence as that encoded by the NMDAR2A-encoding portion of clone NMDA57, deposited with the ATCC under accession number 75442.

- The deposited clone has been deposited at the  
20 American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, U.S.A. 20852, under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples  
25 of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and  
30 all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted. In particular, upon issuance of a U.S. patent based on this or any application  
35 claiming priority to or incorporating this application by

reference thereto, all restriction upon availability of the deposited material will be irrevocably removed.

Exemplary human NMDAR2A subunit-encoding DNAs can alternatively be characterized as those nucleotide sequences which hybridize under high stringency conditions to substantially the entire sequence of Sequence ID No. 10, or substantial portions thereof (i.e., typically at least 25-30 nucleotides thereof), or the NMDAR2A-encoding portion of clone NMDA57 (ATCC accession No. 75442). Especially preferred sequences encoding human NMDAR2A subunits are those which have substantially the same nucleotide sequence as the coding sequence of Sequence ID No. 10, or those which contain substantially the same nucleotide sequence as the coding sequence in the NMDAR2A-encoding portion of clone NMDA57.

Exemplary DNA sequences encoding human NMDAR2B subunits are represented by nucleotides which encode substantially the same amino acid sequence as set forth in Sequence ID No. 14. Exemplary DNAs can alternatively be characterized as those nucleotide sequences which encode a human NMDAR2B subunit and hybridize under high stringency conditions to substantially the entire sequence of Sequence ID No. 13, or substantial portions thereof (i.e., typically at least 25-30 nucleotides thereof). Especially preferred NMDAR2B-encoding sequences are those which have substantially the same nucleotide sequence as the coding sequence in Sequence ID No. 13.

Exemplary DNA sequences encoding human NMDAR2C subunits are represented by nucleotides which encode substantially the same amino acid sequence as set forth in Sequence ID Nos. 6, 6E, 6F, 6G, 6H or 6I.

Exemplary DNAs can alternatively be characterized as those nucleotide sequences which encode a human NMDAR2C

204070-266800

subunit and hybridize under high stringency conditions to substantially the entire sequence of any one of Sequence ID Nos. 5, 5A, 5B, 5C, 5D, 5E, 5F, 5G, 5H, or 5I, or substantial portions thereof (i.e., typically at least 25-30 nucleotides thereof); preferably exemplary DNA will hybridize under high stringency conditions to substantially the entire sequence of any one of Sequence ID Nos. 5, 5E, 5F, or 5G, or substantial portions thereof.

Especially preferred NMDAR2C-encoding sequences are those which have substantially the same nucleotide sequence as the coding sequences in any one of Sequence ID Nos. 5, 5E, 5F, 5G, 5H or 5I; with those having substantially the same sequence as the coding sequences in Sequence ID Nos. 5, 5E, 5F, or 5G being most preferred.

Exemplary DNA sequences encoding human NMDAR2D subunits are represented by nucleotides which encode substantially the same amino acid sequence as set forth in Sequence ID No. 16. Exemplary DNAs can alternatively be characterized as those nucleotide sequences which encode a human NMDAR2D subunit and hybridize under high stringency conditions to substantially the entire sequence of Sequence ID No. 15, or substantial portions thereof (i.e., typically at least 25-30 nucleotides thereof). Especially preferred NMDAR2D-encoding sequences are those which have substantially the same nucleotide sequence as the coding sequence in Sequence ID No. 15.

DNA encoding human NMDA receptor subunits may be isolated by screening suitable human cDNA or human genomic libraries under suitable hybridization conditions with DNA disclosed herein (including nucleotides derived from any of SEQ ID Nos. 1, 1A-1P, 5, 5A-5I, 10, 13 or 15). Suitable libraries can be prepared from neuronal tissue samples, e.g., hippocampus and cerebellum tissue, cell lines, and the like. For example, the library can be screened with a

10038937-010402



portion of DNA including substantially the entire subunit-encoding sequence thereof, or the library may be screened with a suitable probe.

As used herein, a probe is single-stranded DNA or  
5 RNA that has a sequence of nucleotides that includes at least 14 contiguous bases that are the same as (or the complement of) any 14 or more contiguous bases set forth in any of SEQ ID Nos. 1, 1A-1P, 5, 5A-5I, 10, 13 or 15. Preferred regions from which to construct probes include 5'  
10 and/or 3' coding sequences, sequences predicted to encode transmembrane domains, sequences predicted to encode cytoplasmic loops, signal sequences, NMDA binding sites, and the like.

Either the full-length cDNA clones or fragments  
15 thereof can be used as probes, preferably labeled with suitable label means for ready detection. When fragments are used as probes, preferably the DNA sequences will be from the carboxyl end-encoding portion of the DNA, and most preferably will include predicted transmembrane domain-  
20 encoding portions of the DNA sequence (the domains can be predicted based on hydropathy analysis of the deduced amino acid sequence using, for example, the method of Kyte and Doolittle (1982), J. Mol. Biol. Vol. 157:105). These probes can be used, for example, for the identification and  
25 isolation of additional members of the glutamate receptor family.

As a particular application of the invention sequences, genetic screening can be carried out using the nucleotide sequences of the invention as probes. Thus,  
30 nucleic acid samples from patients having neuropathological conditions suspected of involving alteration/modification of any one or more of the glutamate receptors can be screened with appropriate probes to determine if any abnormalities exist with respect to any of the endogenous

glutamate receptors. Similarly, patients having a family history of disease states related to glutamate receptor dysfunction can be screened to determine if they are also predisposed to such disease states.

5 In accordance with another embodiment of the present invention, there is provided a method for identifying DNA encoding human N-methyl-D-aspartate (NMDA) receptor protein subunit(s), said method comprising:

10 contacting human DNA with a nucleic acid probe as described above, wherein said contacting is carried out under high stringency hybridization conditions, and identifying DNA(s) which hybridize to said probe.

After screening the library, positive clones are identified by detecting a hybridization signal; the  
15 identified clones are characterized by restriction enzyme mapping and/or DNA sequence analysis, and then examined, by comparison with the sequences set forth herein to ascertain whether they include DNA encoding a complete NMDA receptor subunit (i.e., if they include translation initiation and  
20 termination codons). If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If the library is genomic, then the overlapping clones may include exons and introns. If the library is a cDNA library, then  
25 the overlapping clones will include an open reading frame. In both instances, complete clones may be identified by comparison with the DNA and encoded proteins provided herein.

Complementary DNA clones encoding various human  
30 NMDA receptor subunits (e.g., NMDAR1, NMDAR2A, NMDAR2B, NMDAR2C, NMDAR2D) have been isolated. Each type of subunit appears to be encoded by a different gene. The DNA clones provided herein may be used to isolate genomic clones encoding each type of subunit and to isolate any splice

variants by screening libraries prepared from different neural tissues. Nucleic acid amplification techniques, which are well known in the art, can be used to locate DNA encoding splice variants of human NMDA receptor subunits.

5 This is accomplished by employing oligonucleotides based on DNA sequences surrounding divergent sequence(s) as primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal the existence of splice variants. Furthermore, isolation of

10 human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns, that correspond to different splice variants of transcripts encoding human NMDA receptor subunits.

It has been found that not all subunits (and

15 variants thereof) are expressed in all neural tissues or in all portions of the brain. Thus, in order to isolate cDNA encoding a particular subunit or splice variants thereof, it is preferable to screen libraries prepared from different neuronal or neural tissues. Preferred tissues to

20 use as sources of nucleic acids for preparing libraries to obtain DNA encoding each subunit include: hippocampus to isolate human NMDAR1-encoding DNAs; hippocampus, cerebellum and fetal brain to isolate NMDAR2-encoding DNAs; and the like.

25 Once DNA encoding a subunit has been isolated, ribonuclease (RNase) protection assays can be employed to determine which tissues express mRNA encoding a particular NMDAR subunit subtype or variant. These assays provide a sensitive means for detecting and quantitating an RNA

30 species in a complex mixture of total cellular RNA. The subunit DNA is labeled and hybridized with cellular RNA. If complementary mRNA is present in the cellular RNA, a DNA-RNA hybrid results. The RNA sample is then treated with RNase, which degrades single-stranded RNA. Any RNA-

35 DNA hybrids are protected from RNase degradation and can be

204070 2655007

visualized by gel electrophoresis and autoradiography. In situ hybridization techniques can also be used to determine which tissues express mRNA encoding a particular NMDAR subunit. The labeled subunit DNAs are hybridized to 5 different brain region slices to visualize subunit mRNA expression.

The distribution of expression of some human NMDA receptor subunits may differ from the distribution of such receptors in rat. For example, RNA encoding the rat 10 NMDAR2C subunit is abundant in rat cerebellum, but is not abundant in rat hippocampus [see, e.g., Monyer et al., Science 256:1217-1221 (1992)]. Numerous human NMDAR2C clones were ultimately obtained, however, from a human hippocampus library. Thus, the distribution of some NMDA 15 receptor subunits in humans and rats appears to be different.

The above-described nucleotide sequences can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete 20 elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the artisan.

An expression vector includes vectors capable of 25 expressing DNAs that are operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or 30 other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that

remain episomal or those which integrate into the host cell genome. Presently preferred plasmids for expression of invention NMDA receptor subunits in eukaryotic host cells, particularly mammalian cells, include cytomegalovirus (CMV) promoter-containing vectors such as pCMV-T7-2 or pCMV-T7-3 (see Figure 6), pMMTVT7(+) or pMMTVT7(-) (modified versions of pMAMneo (Clontech, Palo Alto, CA), prepared as described herein), pCDNA1, and the like.

As used herein, a promoter region refers to a segment of DNA that controls transcription of DNA to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in the practice of the present invention include the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, Moloney murine leukemia virus (MLLV) promoter, and the like.

As used herein, the term "operatively linked" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example, operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes and binds to the promoter, and

transcribes the DNA. In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation (i.e., start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, for example, Kozak (1991) J. Biol. Chem. 266:19867-19870) can be inserted immediately 5' of the start codon and may enhance expression. Likewise, alternative codons, encoding the same amino acid, can be substituted for coding sequences of the NMDAR subunits in order to enhance transcription (e.g., the codon preference of the host cells can be adopted, the presence of G-C rich domains can be reduced, and the like). Furthermore, for potentially enhanced expression of NMDA receptor subunits in amphibian oocytes, the subunit coding sequence can optionally be incorporated into an expression construct wherein the 5'- and 3'-ends of the coding sequence are contiguous with *Xenopus*  $\beta$ -globin gene 5' and 3' untranslated sequences, respectively. For example, NMDA receptor subunit coding sequences can be incorporated into vector pSP64T (see Krieg and Melton (1984) in Nucleic Acids Research 12:7057-7070), a modified form of pSP64 (available from Promega, Madison, WI). The coding sequence is inserted between the 5' end of the  $\beta$ -globin gene and the 3' untranslated sequences located downstream of the SP6 promoter. *In vitro* transcripts can then be generated from the resulting vector. The desirability of (or need for) such modification may be empirically determined.

As used herein, expression refers to the process by which polynucleic acids are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleic acid is derived from genomic DNA,

expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, cytomegalovirus (CMV) promoter-based vectors such as pCMV-T7-2 and pCMV-T7-3 (described herein) or pCDNA1 (Invitrogen, San Diego, CA), and MMTV promoter-based vectors such as pMMTVT7(+) or pMMTVT7(-), described herein.

Full-length DNAs encoding human NMDA receptor subunits have been inserted into vectors pCDNA1, pMMTVT7(+), pCMV-T7-2 and pCMV-T7-3. pCMV-T7-2 is a pUC19-based mammalian cell expression vector containing the CMV promoter/enhancer, SV40 splice/donor sites located immediately downstream of the promoter, a T7 bacteriophage RNA polymerase promoter positioned downstream of the splice sites, followed by an SV40 polyadenylation signal and a polylinker between the T7 promoter and the polyadenylation signal. Placement of NMDA receptor subunit DNA between the CMV promoter and SV40 polyadenylation signal should provide for constitutive expression of the foreign DNA in a mammalian host cell transfected with the construct. Plasmid pCMV-T7-3 is identical to pCMV-T7-2 except that the order of restriction enzyme sites in the polylinker is reversed.

Vectors pMMTVT7(+) and pMMTVT7(-) were prepared by modifying vector pMAMneo (Clontech, Palo Alto, CA). pMAMneo is a mammalian expression vector that contains the Rous Sarcoma Virus (RSV) long terminal repeat (LTR) enhancer, linked to the dexamethasone-inducible mouse mammary tumor virus (MMTV)-LTR promoter, followed by SV40 splicing and polyadenylation sites. pMAMneo also contains

the *E. coli* neo gene for selection of transformants, as well as the  $\beta$ -lactamase gene (encoding a protein which imparts ampicillin-resistance) for propagation in *E. coli*.

Vector pMMTVT7(+) can be generated by  
 5 modification of pMAMneo to remove the neo gene and insert the multiple cloning site and T7 and T3 promoters from pBluescript (Stratagene, La Jolla, CA). Thus, pMMTVT7(+) contains the RSV-LTR enhancer linked to the MMTV-LTR promoter, a T7 bacteriophage RNA polymerase promoter  
 10 positioned downstream of the MMTV-LTR promoter, a polylinker positioned downstream of the T7 promoter, a T3 bacteriophage RNA polymerase promoter positioned downstream of the T7 promoter, and SV40 splicing and polyadenylation sites positioned downstream of the T3 promoter. The  
 15  $\beta$ -lactamase gene (encoding a protein which imparts ampicillin-resistance) from pMAMneo is retained in pMMTVT7(+), although it is incorporated in the reverse orientation relative to the orientation in pMAMneo.

Vector pMMTVT7(-) is identical to pMMTVT7(+)  
 20 except that the positions of the T7 and T3 promoters are switched, i.e., the T3 promoter in pMMTVT7(-) is located where the T7 promoter is located in pMMTVT7(+), and the T7 promoter in pMMTVT7(-) is located where the T3 promoter is located in pMMTVT7(+). Therefore, vectors pMMTVT7(+) and  
 25 pMMTVT7(-) contain all of the regulatory elements required for expression of heterologous DNA in a mammalian host cell, wherein the heterologous DNA has been incorporated into the vectors at the polylinker. In addition, because the T7 and T3 promoters are located on either side of the  
 30 polylinker, these plasmids can be used for synthesis of *in vitro* transcripts of heterologous DNA that has been subcloned into the vectors at the polylinker.

For inducible expression of human NMDA receptor subunit-encoding DNA in a mammalian cell, the DNA can be

10038937.040403



inserted into a plasmid such as pMMTVT7(+) or pMMTVT7(-). These plasmids contain the mouse mammary tumor virus (MMTV) promoter for steroid-inducible expression of operatively associated foreign DNA. If the host cell does not express endogenous glucocorticoid receptors required for uptake of glucocorticoids (i.e., inducers of the MMTV promoter) into the cell, it is necessary to additionally transfect the cell with DNA encoding the glucocorticoid receptor (ATCC accession no. 67200). For synthesis of *in vitro* transcripts, full-length human DNA clones encoding human NMDAR1, NMDAR2A, NMDAR2B, NMDAR2C and NMDAR2D can also be subcloned into pIBI24 (International Biotechnologies, Inc., New Haven, CT), pCMV-T7-2, pCMV-T7-3, pMMTVT7(+), pMMTVT7(-), pBluescript (Stratagene, La Jolla, CA) or pGEM7Z (Promega, Madison, WI).

In accordance with another embodiment of the present invention, there are provided cells containing the above-described polynucleic acids (i.e., DNA or mRNA). Such host cells as bacterial, yeast and mammalian cells can be used for replicating DNA and producing NMDA receptor subunit(s). Methods for assessing receptor expression and function are described in PCT Application Nos. PCT/US91/05625 and PCT/US92/11090, and in co-pending U.S. Application Serial Nos. 07/563,751 and 07/812,254. The subject matter of these documents is hereby incorporated by reference herein in their entirety.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press). Heterologous DNA may be introduced into host cells by any method known to those of skill in the

art, such as transfection with a vector encoding the heterologous DNA by  $\text{CaPO}_4$  precipitation (see, e.g., Wigler et al. (1979) Proc. Natl. Acad. Sci. 76:1373-1376) or lipofectamine (GIBCO BRL #18324-012). Recombinant cells can then be cultured under conditions whereby the subunit(s) encoded by the DNA is (are) expressed. Preferred cells include mammalian cells (e.g., HEK293, CHO, BHKBI and Ltk<sup>-</sup> cells, mouse monocyte macrophage P388D1 and J774A-1 cells (available from ATCC, Rockville, MD), and the like), yeast cells (e.g., methylotrophic yeast cells, such as *Pichia pastoris*), bacterial cells (e.g., *Escherichia coli*), and the like.

While the DNA provided herein may be expressed in any eukaryotic cell, including yeast cells (such as, for example, *P. pastoris* (see U.S. Patent Nos. 4,882,279, 4,837,148, 4,929,555 and 4,855,231), *Saccharomyces cerevisiae*, *Candida tropicalis*, *Hansenula polymorpha*, and the like), mammalian expression systems, including commercially available systems and other such systems known to those of skill in the art, for expression of DNA encoding the human NMDA receptor subunits provided herein are presently preferred. *Xenopus* oocytes are preferred for expression of *in vitro* RNA transcripts of the DNA.

In preferred embodiments, human NMDAR subunit-encoding DNA is ligated into a vector, and introduced into suitable host cells to produce transformed cell lines that express a specific human NMDA receptor subtype, or specific combinations of subunits. The resulting cell lines can then be produced in quantity for reproducible quantitative analysis of the effects of known or potential drugs on receptor function. In other embodiments, mRNA may be produced by *in vitro* transcription of DNA encoding each subunit. This mRNA, either from a single subunit clone or from a combination of clones, can then be injected into *Xenopus* oocytes where the mRNA directs the synthesis of the

human receptor subunits, which then form functional receptors. Alternatively, the subunit-encoding DNA can be directly injected into oocytes for expression of functional receptors. The transfected mammalian cells or injected  
 5 oocytes may then be used in the methods of drug screening provided herein.

Eukaryotic cells in which DNA or RNA may be introduced include any cells that are transfectable by such DNA or RNA or into which such DNA or RNA may be injected.  
 10 Preferred cells are those that can be transiently or stably transfected and also express the DNA and RNA. Presently most preferred cells are those that can form recombinant or heterologous human NMDA receptors comprising one or more subunits encoded by the heterologous DNA. Such cells may  
 15 be identified empirically or selected from among those known to be readily transfected or injected.

Exemplary cells for introducing DNA include cells of mammalian origin (e.g., COS cells, mouse L cells, Chinese hamster ovary (CHO) cells, human embryonic kidney  
 20 (HEK) cells (particularly HEK293 cells that can be frozen in liquid nitrogen and then thawed and regrown; for example, those described in U.S. Patent No. 5,024,939 to Gorman (see, also, Stillman et al. (1985) Mol. Cell. Biol. 5:2051-2060)), African green monkey cells and other such  
 25 cells known to those of skill in the art), amphibian cells (e.g., *Xenopus laevis* oocytes), yeast cells (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*), and the like. Exemplary cells for expressing injected RNA transcripts include *Xenopus laevis* oocytes. Cells that are preferred  
 30 for transfection of DNA are known to those of skill in the art or may be empirically identified, and include HEK293 (which are available from ATCC under accession #CRL 1573); Ltk<sup>-</sup> cells (which are available from ATCC under accession #CCL1.3); COS-7 cells (which are available from ATCC under  
 35 accession #CRL 1651); and DG44 cells (dhfr<sup>-</sup> CHO cells; see,

204070-2688E007

e.g., Urlaub et al. (1986) Cell. Molec. Genet. 12: 555). Presently preferred cells include Ltk<sup>-</sup> cells and DG44 cells.

DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art.

5 Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene (such as, for example, the gene for thymidine kinase, dihydrofolate reductase, neomycin resistance, and the like), and growing the transfected

10 cells under conditions selective for cells expressing the marker gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene (such as the *E. coli*  $\beta$ -galactosidase gene) to monitor transfection efficiency. Selectable marker genes are not included in

15 the transient transfections because the transfectants are typically not grown under selective conditions, and are usually analyzed within a few days after transfection.

To produce such stably or transiently transfected cells, the cells should be transfected with a sufficient

20 concentration of subunit-encoding nucleic acids to form human NMDA receptors that contain the human subunits encoded by heterologous DNA. The precise amounts and ratios of DNA encoding the subunits may be empirically determined and optimized for a particular combination of

25 subunits, cells and assay conditions. Recombinant cells that express NMDA receptors containing subunits encoded only by the heterologous DNA or RNA are especially preferred.

Heterologous DNA may be maintained in the cell as

30 an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Methods for transfection, injection and culturing

recombinant cells are known to the skilled artisan. Similarly, the human NMDA receptor subunits may be purified using protein purification methods known to those of skill in the art. For example, antibodies or other ligands that  
 5 specifically bind to one or more of the subunits may be used for affinity purification and immunoprecipitation of the subunit or human NMDA receptors containing the subunits.

As used herein, heterologous or foreign DNA and  
 10 RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome of the cell in which it is present or to DNA or RNA which is found in a location or locations in the genome that differ from that in which it occurs in nature. Typically, heterologous or  
 15 foreign DNA and RNA refers to DNA or RNA that is not endogenous to the host cell and has been artificially introduced into the cell. Examples of heterologous DNA include DNA that encodes a human NMDA receptor subunit, DNA that encodes RNA or proteins that mediate or alter  
 20 expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes, and the like. The cell that expresses heterologous DNA may contain DNA encoding the same or different expression products. Heterologous DNA need not be expressed and may  
 25 be integrated into the host cell genome or maintained episomally.

Recombinant receptors on recombinant eukaryotic cell surfaces may contain one or more subunits encoded by the DNA or mRNA encoding human NMDA receptor subunits, or  
 30 may contain a mixture of subunits encoded by the host cell and subunits encoded by heterologous DNA or mRNA. Recombinant receptors may be homomeric or may be a heteromeric combination of multiple subunits. Mixtures of DNA or mRNA encoding receptors from various species, such  
 35 as rats and humans, may also be introduced into the cells.

Thus, a cell can be prepared that expresses recombinant receptors containing only NMDAR1 subunits, or a combination of any one or more NMDAR1 and any one or more NMDAR2 subunits provided herein. For example, NMDAR1 subunits of the present invention can be co-expressed with NMDAR2A, NMDAR2B, NMDAR2C and/or NMDAR2D receptor subunits. Specific examples of heteromeric combinations of recombinant human NMDAR subunits that have been expressed in *Xenopus* oocytes include NMDAR1 + NMDAR2A, NMDAR1 + NMDAR2B, and NMDAR1 + NMDAR2A + NMDAR2C (see Example 9).

The DNA, mRNA, vectors, receptor subunits, receptor subunit combinations and cells provided herein permit production of selected NMDA receptor subunits and specific combinations thereof, as well as antibodies to said receptor subunits. This provides a means to prepare synthetic or recombinant receptors and receptor subunits that are substantially free of contamination from many other receptor proteins whose presence can interfere with analysis of a single NMDA receptor subtype. The availability of desired receptor subtypes makes it possible to observe the effect of a drug substance on a particular receptor subtype or combination of NMDA receptor subunits, and to thereby perform initial *in vitro* screening of the drug substance in a test system that is specific for humans and specific for a human NMDA receptor subtype or combination of NMDA receptor subunits. The availability of specific antibodies makes it possible to identify the subunit combinations expressed *in vivo*. Such specific combinations can then be employed as preferred targets in drug screening.

The ability to screen drug substances *in vitro* to determine the effect of the drug on specific receptor compositions should permit the development and screening of receptor subtype-specific or disease-specific drugs. Also, testing of single receptor subunits or specific

combinations of various types of receptor subunits with a variety of potential agonists or antagonists provides additional information with respect to the function and activity of the individual subunits and should lead to the identification and design of compounds that are capable of very specific interaction with one or more types of receptor subunits or receptor subtypes. The resulting drugs should exhibit fewer unwanted side effects than drugs identified by screening with cells that express a variety of receptor subtypes.

Further in relation to drug development and therapeutic treatment of various disease states, the availability of DNAs encoding human NMDA receptor subunits enables identification of any alterations in such genes (e.g., mutations) which may correlate with the occurrence of certain disease states. In addition, the creation of animal models of such disease states becomes possible, by specifically introducing such mutations into synthetic DNA sequences which can then be introduced into laboratory animals or *in vitro* assay systems to determine the effects thereof.

In another aspect, the invention comprises functional peptide fragments, and functional combinations thereof, encoded by the DNAs of the invention. Such functional peptide fragments can be produced by those skilled in the art, without undue experimentation, by eliminating some or all of the amino acids in the sequence not essential for the peptide to function as a glutamate receptor. A determination of the amino acids that are essential for glutamate receptor function is made, for example, by systematic digestion of the DNAs encoding the peptides and/or by the introduction of deletions into the DNAs. The modified (e.g., deleted or digested) DNAs are expressed, for example, by transcribing the DNA and then introducing the resulting mRNA into *Xenopus* oocytes, where

translation of the mRNAs will occur. Functional analysis of the proteins thus expressed in the oocytes is accomplished by exposing the oocytes to ligands known to bind to and functionally activate glutamate receptors, and  
5 then monitoring the oocytes to see if the expressed fragments form ion channel(s). If ion channel(s) are detected, the fragments are functional as glutamate receptors.

The above-described method can be carried out in  
10 the presence of NMDAR1-like receptor subunits alone, or in the presence of combinations of NMDAR1-like and NMDAR2-like receptor subunits. Thus, for example, when the protein being tested is an NMDAR2-like receptor subunit, the additional subunit is preferably an NMDAR1-like subunit.

15 In accordance with still another embodiment of the present invention, there is provided a method for identifying compounds which bind to human N-methyl-D-aspartate (NMDA) receptor subunit(s), said method comprising employing receptor proteins of the invention in  
20 a competitive binding assay. Such an assay can accommodate the rapid screening of a large number of compounds to determine which compounds, if any, are capable of binding to NMDA receptors. Subsequently, more detailed assays can be carried out with those compounds found to bind, to  
25 further determine whether such compounds act as modulators, agonists or antagonists of invention receptors.

Another application of the binding assay of the invention is the assay of test samples (e.g., biological fluids) for the presence or absence of receptors of the  
30 present invention. Thus, for example, serum from a patient displaying symptoms related to glutamatergic pathway dysfunction can be assayed to determine if the observed symptoms are perhaps caused by over- or under-production of such receptor(s).

204070 ZEEB001



The binding assays contemplated by the present invention can be carried out in a variety of ways, as can readily be identified by those of skill in the art. For example, competitive binding assays can be employed, such as radioreceptor assays, and the like.

In accordance with a further embodiment of the present invention, there is provided a bioassay for identifying compounds which modulate the activity of human NMDA receptors of the invention, said bioassay comprising:

- 10 (a) exposing cells containing DNA encoding human NMDA receptor subunit(s), wherein said cells express functional NMDA receptors, to at least one compound whose ability to modulate the ion channel activity of said receptors is sought to be determined; and thereafter
- 15 (b) monitoring said cells for changes in ion channel activity.

The above-described bioassay enables the identification of agonists and antagonists for human NMDA receptors. According to this method, recombinant NMDA receptors are contacted with an "unknown" or test substance (in the further presence of a known NMDA agonist, when antagonist activity is being tested), the ion channel activity of the known glutamate receptor is monitored subsequent to the contact with the "unknown" or test substance, and those substances which increase or decrease the ion channel response of the known glutamate receptor(s) are identified as functional ligands (i.e., modulators, agonists or antagonists) for human NMDA receptors.

30 In accordance with a particular embodiment of the present invention, recombinant human NMDA receptor-expressing mammalian cells or oocytes can be contacted with a test compound, and the modulating effect(s) thereof can then be evaluated by comparing the

204070 2E68E00T

NMDA receptor-mediated response in the presence and absence of test compound, or by comparing the response of test cells, or control cells (i.e., cells that do not express NMDA receptors), to the presence of the compound.

5           As used herein, a compound or signal that  
"modulates the activity of an NMDA receptor" refers to a  
compound or signal that alters the activity of NMDA  
receptors so that activity of the NMDA receptor is  
different in the presence of the compound or signal than in  
10 the absence of the compound or signal. In particular, such  
compounds or signals include agonists and antagonists. The  
term agonist refers to a substance or signal, such as NMDA,  
that activates receptor function; and the term antagonist  
refers to a substance that interferes with receptor  
15 function. Typically, the effect of an antagonist is  
observed as a blocking of activation by an agonist.  
Antagonists include competitive and non-competitive  
antagonists. A competitive antagonist (or competitive  
blocker) interacts with or near the site specific for the  
20 agonist (e.g., ligand or neurotransmitter). A non-  
competitive antagonist or blocker inactivates the  
functioning of the receptor by interacting with a site  
other than the site that interacts with the agonist.

As understood by those of skill in the art, assay  
25 methods for identifying compounds that modulate human NMDA  
receptor activity (e.g., agonists and antagonists)  
generally require comparison to a control. One type of a  
"control" cell or "control" culture is a cell or culture  
that is treated substantially the same as the cell or  
30 culture exposed to the test compound, except the control  
culture is not exposed to test compound. For example, in  
methods that use voltage clamp electrophysiological  
procedures, the same cell can be tested in the presence and  
absence of test compound, by merely changing the external  
35 solution bathing the cell. Another type of "control" cell

or "control" culture may be a cell or a culture of cells which is identical to the transfected cells, except the cells employed for the control culture do not express functional human NMDA receptor subunits. In this  
5 situation, the response of test cell to test compound is compared to the response (or lack of response) of receptor-negative (control) cell to test compound, when cells or cultures of each type of cell are exposed to substantially the same reaction conditions in the presence of compound  
10 being assayed.

In accordance with yet another embodiment of the present invention, the ion channel activity of human N-methyl-D-aspartate (NMDA) receptors can be modulated by contacting such receptors with an effective amount of at  
15 least one compound identified by the above-described bioassay.

In accordance with yet another embodiment of the present invention, there are provided antibodies generated against the above-described receptor proteins. Such  
20 antibodies can be employed for studying receptor tissue localization, subunit composition, structure of functional domains, as well as in diagnostic applications, therapeutic applications, and the like. Preferably, for therapeutic applications, the antibodies employed will be monoclonal  
25 antibodies.

The above-described antibodies can be prepared employing standard techniques, as are well known to those of skill in the art, using the invention receptor proteins or portions thereof as antigens for antibody production.  
30 Both anti-peptide and anti-fusion protein antibodies can be used [see, for example, Bahouth et al. (1991) Trends Pharmacol Sci. vol. 12:338-343; Current Protocols in Molecular Biology (Ausubel et al., eds.) John Wiley and Sons, New York (1989)]. Factors to consider in selecting

portions of the NMDAR subunits for use as immunogen (as either a synthetic peptide or a recombinantly produced bacterial fusion protein) include antigenicity, accessibility (i.e., extracellular and cytoplasmic domains), uniqueness to the particular subunit, etc.

The availability of subunit-specific antibodies makes possible the application of the technique of immunohistochemistry to monitor the distribution and expression density of various subunits (e.g., in normal vs diseased brain tissue). Such antibodies could also be employed for diagnostic and therapeutic applications.

In accordance with still another embodiment of the present invention, there are provided methods for modulating the ion channel activity of receptor(s) of the invention by contacting said receptor(s) with an effective amount of the above-described antibodies.

The antibodies of the invention can be administered to a subject employing standard methods, such as, for example, by intraperitoneal, intramuscular, intravenous, or subcutaneous injection, implant or transdermal modes of administration, and the like. One of skill in the art can readily determine dose forms, treatment regimens, etc, depending on the mode of administration employed.

The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1Isolation of DNA encoding human NMDA receptor  
NMDAR1 subunitsA. cDNA Library Screening

5 RNA isolated from human hippocampus tissue was used as a template for the synthesis of oligo dT-primed and randomly primed, single-stranded cDNA according to standard procedures [see, for example, Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor  
10 Laboratory Press, Cold Spring Harbor, NY]. The single-stranded cDNA was converted to double-stranded cDNA, and *EcoRI*/*SnaBI*/*XhoI* adaptors were added to the ends thereof. The cDNAs were separated by size using agarose gel electrophoresis, and those that were >2.0 kb were ligated  
15 into *EcoRI*-digested  $\lambda$ gt10 bacteriophage vectors. The resulting cDNA library was amplified by replication of each clone through limited infection of a bacterial host, and stored at -70°C.

The amplified hippocampus oligo dT-primed cDNA  
20 library was later retrieved from storage and  $1 \times 10^6$  recombinants were screened for hybridization to oligonucleotides corresponding to nucleotides 96-128 (SE7) and nucleotides 2576-2609 (SE8) of the rat NMDAR1A receptor cDNA (see Moriyoshi et al. (1991) *Nature* 354:31).  
25 Hybridization was performed at 42°C in 6X SSPE, 5X Denhart's solution, 10% formamide, 0.2% SDS and 200  $\mu$ g/ml herring sperm DNA. Washes were performed in 1X SSPE and 0.2% SDS at 50°C. Hybridizing clones (e.g. NMDA1-3) were identified. These clones hybridized to SE8 but not to SE7.

30 A randomly primed primary human hippocampus cDNA library ( $\sim 2 \times 10^5$  recombinants prepared by selecting only cDNAs >2.0 kb for inclusion in the library) was screened under the same conditions for hybridization to

oligonucleotide SE8 and an oligonucleotide corresponding to nucleotides 129-141 of the rat NMDAR1A receptor cDNA (SE11). Five hybridizing clones, which hybridized to SE8 and not to SE11, were identified: NMDA5-7 and NMDA10-11.

5 B. Characterization of Clones

The clones were plaque purified and characterized by restriction enzyme mapping and DNA sequence analysis of the inserts. One of the clones, NMDA11 (see Sequence ID No. 1B for a description of a portion of NMDA11), is a full-length cDNA (i.e., it contains translation initiation and termination codons) encoding a complete NMDAR1 subunit. The remaining clones are partial cDNAs. Clones NMDA2, NMDA3 (see Sequence ID No. 1D), NMDA5, NMDA6, NMDA7 (see Sequence ID No. 1C), and NMDA10 (see Sequence ID No. 1A for a description of a portion of NMDA10) contain a translation termination codon but lack nucleotides at the 5' end of the coding sequence.

Characterization of the clones revealed that the isolated cDNAs correspond to different alternatively spliced forms of the human NMDAR1 subunit transcript. The four types of alternate splicing represented by the clones are depicted schematically in Figure 1. Clone NMDA10 (which lacks 5' untranslated sequences as well as 60 nucleotides of the 5' end of the coding sequence) is used as a reference to which the other variants are compared. Clone NMDA11 lacks 363 nucleotides (in the 3' portion of the clone) that are present in NMDA10. This 363-nucleotide deletion does not disrupt the reading frame of the transcript; however, it results in a different termination codon. The last 69 nucleotides of the coding sequence of NMDA11 correspond to 3' untranslated sequence of clone NMDA10 (i.e., nucleotides 3325-3393 of Sequence ID No. 1). Clone NMDA7 lacks the same 363-nucleotide sequence that is deleted from NMDA11; however, NMDA7 further lacks 204

204070 263E007

nucleotides at the 5' end that are present in NMDA10 and NMDA11. This 204-nucleotide deletion also does not disrupt the reading frame of the transcript. Additionally, NMDA7 contains a 63-nucleotide in-frame insertion at the 5' end relative to NMDA10 and NMDA11. The last 69 base pairs of the coding sequence of NMDA7 correspond to 3' untranslated sequence of NMDA10 i.e., nucleotides 3325-3393 of Sequence ID No. 1). Clone NMDA3 lacks 1087 base pairs at the 3' end that are present in NMDA10. This 1087-base pair deletion does not disrupt the reading frame of the transcript; however it results in a different termination codon. The last 231 base pairs of the coding sequence of NMDA3 correspond to 3' untranslated sequence of clone NMDA10 (i.e., nucleotides 4049-4279 in Sequence ID No. 1).

15

Example 2Preparation of full-length NMDAR1 subunit cDNA constructs

Portions of clones NMDA10, NMDA11, NMDA7 and NMDA3 were ligated together to construct full-length cDNAs encoding variants of the NMDA receptor NMDAR1 subunit. The full-length NMDAR1 subunit cDNAs were incorporated into vector pcDNA1 (Invitrogen, San Diego, CA) for use in expressing the receptor subunits in mammalian host cells and for use in generating *in vitro* transcripts of the DNAs to be expressed in *Xenopus* oocytes.

25

Vector pcDNA1 is a pUC19-based plasmid that contains the following elements in the 5'-to-3' order: the cytomegalovirus (CMV) immediate early gene promoter/enhancer, the bacteriophage T7 RNA polymerase promoter, a polylinker, the bacteriophage SP6 RNA polymerase promoter, SV40 RNA processing (i.e., splice donor/acceptor) signals, SV40 polyadenylation signal, and the ColE1 origin and supF suppressor tRNA to permit maintenance of the vector in *Escherichia coli* strains with the P3 episome. This vector thus contains all the

1003333 0410402  
204070 2668007

regulatory elements required for expression of heterologous DNA in a mammalian host cell, wherein the heterologous DNA has been incorporated into the vector at the polylinker. In addition, because the T7 and SP6 promoters are located  
 5 on either side of the polylinker, this plasmid can be used for synthesis of *in vitro* transcripts of heterologous DNA that has been subcloned into the vector at the polylinker.

A. NMDAR1A

Full-length construct NMDAR1A was prepared by  
 10 ligation of a 5' portion of NMDA11 (beginning 5' of the translation initiation codon and extending to the *HindIII* site in the middle of the clone) and a 3' portion of NMDA10 (beginning at the *HindIII* site in the middle of the clone and extending 3' of the translation termination codon) as  
 15 depicted in Figure 2. The two DNA fragments were joined in mammalian expression vector pcDNA1.

Initially, the strategy for generating the NMDAR1 construct involved a first step of separately subcloning the entire 4.0 kb *EcoRI* insert fragment of NMDA10 and the  
 20 entire 4.0 kb *SnaBI* insert fragment of NMDA11 into pcDNA1; however, two attempts employing this cloning strategy were unsuccessful. It appeared that there may have been selection against *E. coli* hosts retaining the complete insert fragments since the surviving recombinant *E. coli*  
 25 that were analyzed contained incomplete insert cDNAs from which nucleotides had been deleted. Therefore, it was necessary to prepare the full-length NMDAR1A construct in several steps by subcloning and combining various fragments of NMDA10 and NMDA11 in pcDNA1 as follows (see Figure 3 for  
 30 locations of restriction enzyme sites).

Clone NMDA10 was digested with *BglIII* and *EcoRI* and the ~3.3 kb fragment containing nucleotides 1020-4298 of Sequence ID No. 1 was isolated and subcloned into

10030937.010402



*Bam*HI/*Eco*RI-digested pcDNA1. The resulting plasmid was digested with *Hind*III and *Nhe*I and the fragment containing nucleotides 2137-4298 of Sequence ID No. 1 plus a portion of pcDNA1 was isolated.

5           Clone NMDA11 was digested with *Eco*RI and *Hind*III and the ~2.1 kb fragment containing nucleotides 1-2136 of Sequence ID No. 1 was isolated and subcloned into *Eco*RI/*Hind*III-digested modified pcDNA1 (modified by deletion of the *Hind*III site located 5' of the *Eco*RI site  
10 in the polylinker and addition of a *Hind*III site into the polylinker at a position 3' of the *Eco*RI site). The resulting plasmid was digested with *Nhe*I and *Hind*III and the fragment containing nucleotides 1-2136 of Sequence ID No. 1 plus a portion of modified pcDNA1 was isolated. This  
15 *Nhe*I/*Hind*III fragment was then ligated to the *Hind*III/*Nhe*I fragment containing nucleotides 2137-4298 of Sequence ID No. 1 to generate the full-length construct NMDAR1A (see Figure 2). The ligation mix was used to transform *E. coli* strain MC1061/P3. Because the *Nhe*I site in pcDNA1 occurs  
20 within the supF selection gene, only *E. coli* containing the correctly ligated, complete NMDAR1A plasmid (which has the complete, functional selection gene) were able to survive the selection process. This fragment subcloning strategy enabled selection of the desired correct NMDAR1A-containing  
25 *E. coli* host cells, even though the total number of such recombinant host cells was small.

          In summary, construct NMDAR1A contains 261 base pairs of 5' untranslated sequence from NMDAR11 (nucleotides 1-261 of Sequence ID No. 1) and a complete coding sequence  
30 (nucleotides 262-3078 of Sequence ID No.1) for the NMDAR1A variant of the NMDAR1 subunit as well as 1220 base pairs of 3' untranslated sequence (nucleotides 3079-4298 of Sequence ID No. 1). The NMDAR1A-encoding sequence is operatively

204079-4298

linked to the regulatory elements in pcDNA1 for expression in mammalian cells.

B. NMDAR1-Δ363

Full-length construct NMDAR1-Δ363 was prepared by ligation of a 5' portion of NMDA11 (beginning 5' of the translation initiation codon and extending to the *HindIII* site in the middle of the clone, i.e., nucleotides 1-2136 in Sequence ID No. 1) and a 3' portion of NMDA11 (beginning at the *HindIII* site in the middle of the clone and extending 3' of the translation termination codon, i.e., nucleotides 2137-2961 and 3325-4298 of Sequence ID No. 1). As described above, due to the difficulty in directly subcloning the entire 4.0 kb *SnaBI* NMDA11 insert into pcDNA1, it was necessary to generate the construct by ligating two fragments of the NMDA11 insert into pcDNA1 as follows (see Figure 3 for locations of restriction enzyme sites).

To obtain the 5' NMDA11 fragment, clone NMDA11 was digested with *EcoRI* and *HindIII* and the ~2.2 kb fragment containing nucleotides 1-2136 of Sequence ID No. 1 was isolated and subcloned into *EcoRI/HindIII*-digested modified pcDNA1 (modified as described above). The resulting plasmid was digested with *NheI* and *HindIII* and the fragment containing nucleotides 1-2136 of Sequence ID No. 1 plus a portion of modified pcDNA1 was isolated.

To obtain the 3' NMDA11 fragment, clone NMDA11 was digested with *BglIII* and *EcoRI* and the 3.0 kb fragment containing nucleotides 1020-2961 and 3325-4298 of Sequence ID No. 1 was isolated and subcloned into *BamHI/EcoRI*-digested pcDNA1. The resulting plasmid was digested with *HindIII* and *NheI* and the fragment containing nucleotides 2137-2961 and 3325-4298 of Sequence ID No. 1 plus a portion of pcDNA1 was isolated. This *HindIII/NheI* fragment was

2010T9 255937 010102

then ligated to the *NheI/HindIII* fragment containing nucleotides 1-2136 of Sequence ID No. 1 to generate NMDAR1-Δ363.

In summary, construct NMDAR1-Δ363 contains 261  
 5 base pairs of 5' untranslated sequence (nucleotides 1-261 of Sequence ID No. 1) and a complete coding sequence for the NMDAR1-Δ363 variant NMDAR1 subunit (nucleotides 262-2961 and 3325-3393 of Sequence ID No. 1) as well as 905  
 10 base pairs of 3' untranslated sequence (nucleotides 3394-4298 of Sequence ID No. 1). Thus, NMDAR1-Δ363 differs from NMDAR1 in that it lacks 363 nucleotides (nucleotides 2962-3324 of Sequence ID No. 1) that comprise the last 117 nucleotides of the coding sequence and the first 246 nucleotides of the 3' untranslated sequence of NMDAR1. The  
 15 NMDAR1-Δ363 subunit variant-encoding sequence is operatively linked to the regulatory elements in pCDNA1 for expression in mammalian cells.

#### C. NMDAR1-Δ1087

Full-length construct NMDAR1-Δ1087 was prepared  
 20 by replacing the 3' end of the NMDAR1 variant-encoding insert of NMDAR1-Δ363 with a fragment from the 3' end of clone NMDA3 (see Figure 2). Plasmid NMDAR1-Δ363 was partially digested with *PstI* and completely digested with *XbaI*. There is a *PstI* site ~112 nucleotides upstream of  
 25 the location of the 363-nucleotide deletion in NMDAR1-Δ363 and an *XbaI* site in the polylinker located downstream of the 3' untranslated sequence of NMDAR1-Δ363 (see Figure 3). Thus, *PstI/XbaI* digestion of NMDAR1-Δ363 results in removal of a fragment containing nucleotides 2850-2961 and 3325-  
 30 4298 of Sequence ID No. 1 from the vector. The larger fragment was isolated from the digest.

The insert of clone NMDA3 was cloned into the *EcoRI* restriction site(s) of pGEM (Promega, Madison, WI);

204070-2288007

and the resulting plasmid was digested with *Pst*I and *Xba*I. The smaller fragment containing nucleotides 2850-2961 and 4049-4298 of Sequence ID No. 1 was isolated and ligated to the larger fragment from the *Pst*I/*Xba*I digest of NMDAR1-  
 5  $\Delta$ 363. The resulting construct was designated NMDAR1- $\Delta$ 1087.

In summary, NMDAR1- $\Delta$ 1087 contains 261 base pairs of 5' untranslated sequence (nucleotides 1-261 in Sequence ID No. 1), the complete coding sequence for the NMDAR1- $\Delta$ 1087 variant NMDAR1 subunit (nucleotides 262-2961 and  
 10 4049-4279 of Sequence ID No. 1) and 19 base pairs of 3' untranslated sequence (nucleotides 4280-4298 of Sequence ID No. 1). Thus, NMDAR1- $\Delta$ 1087 differs from NMDAR1 in that it lacks 1087 nucleotides (nucleotides 2962-4048 of Sequence ID No. 1) that comprise the last 117 nucleotides of the  
 15 coding sequence and the first 970 nucleotides of the 3' untranslated sequence of NMDAR1. The NMDAR1- $\Delta$ 1087 subunit variant-encoding sequence is operatively linked to the regulatory elements in pcDNA1 for expression in mammalian cells.

#### 20 D. NMDAR1-I63- $\Delta$ 204

Full-length construct NMDAR1-I63- $\Delta$ 204 was prepared by replacing a 1399-nucleotide fragment of construct NMDAR1A (i.e., nucleotides 738-2136 of Sequence ID No. 1) with the *Pvu*II-*Hind*III fragment of NMDA7 (i.e.,  
 25 nucleotides 738-831 of sequence ID No. 1, plus nucleotides 1-63 of Sequence ID No. 3 and nucleotides 832-984 and 1189-2136 of Sequence ID No. 1), as depicted in Figure 2. Because there are multiple *Pvu*II sites in the NMDAR1 construct, a several-step process was required for  
 30 construction of NMDAR1-I63- $\Delta$ 204 as follows (see Figure 3 for the location of restriction enzyme sites).

The ~2.2-kb *Eco*RI-*Hind*III fragment isolated from construct NMDAR1A and containing nucleotides 1-2136 of

20101010 10030937 010102

Sequence ID No. 1 was ligated with modified pcDNA1 (modified as described in Example 2A) that had been digested with *EcoRI* and *HindIII*. The resulting plasmid was digested with *AvrII* and self-ligated to remove two *PvuII* sites from a portion of the plasmid contributed by pcDNA1. The plasmid was then partially digested with *PvuII* and completely digested with *HindIII*. The digest was ligated with a 1258-nucleotide *PvuII-HindIII* fragment isolated from clone NMDA7. The resulting plasmid, designated NMDAR1-I63- $\Delta$ 204-5', was digested with *BamHI* and *HindIII* and the ~2-kb fragment containing nucleotides 1-831 of Sequence ID No. 1, plus nucleotides 1-63 of Sequence ID No. 3 and nucleotides 832-984 and 1189-2136 of Sequence ID No. 1 was isolated and ligated to *BamHI/HindIII*-digested NMDAR1 to generate NMDAR1-I63- $\Delta$ 204.

NMDAR1-I63- $\Delta$ 204 contains 261 base pairs of 5' untranslated sequence (nucleotides 1-261 in Sequence ID No. 1), the complete coding sequence for the NMDAR1-I63- $\Delta$ 204 variant NMDAR1 subunit (nucleotides 262-831 of Sequence ID No. 1 plus nucleotides 1-63 of Sequence ID No. 3 and nucleotides 832-984 and 1189-3078 of Sequence ID No. 1) and 1220 base pairs of 3' untranslated sequence (nucleotides 3079-4298 of Sequence ID No. 1). Thus NMDAR1-I63- $\Delta$ 204 differs from NMDAR1 in that it contains 63 nucleotides that are not present in NMDAR1 (nucleotides 1-63 of Sequence ID No. 3) located between nt 831 and 832 of Sequence ID No. 1. Further, NMDAR1-I63- $\Delta$ 204 lacks 204 nucleotides that are present in NMDAR1 (nucleotides 985-1188 of Sequence ID No. 1). The NMDAR1-I63- $\Delta$ 204 subunit variant-encoding sequence is operatively linked to the regulatory elements in pcDNA1 for expression in mammalian cells.

E. NMDAR1-I63

Full-length construct NMDAR1-I63 can be described as NMDAR1 in which a 173-bp fragment (nucleotides 738-910 of Sequence ID No. 1) is replaced with the 236-bp PvuII-SmaI fragment of NMDA7 (nucleotides 738-831 of Sequence ID No. 1, plus nucleotides 1-63 of Sequence ID No. 3 and nucleotides 832-910 of Sequence ID No. 1). Because there are multiple PvuII sites in the NMDAR1 construct, a several-step process was required for construction of NMDAR1-I63 as follows. Plasmid NMDAR1-I63- $\Delta$ 204-5' was partially digested with SmaI and completely digested with HindIII. The larger vector fragment was ligated with the 1226-bp SmaI/HindIII fragment isolated from NMDA11 (nucleotides 911-2136 of Sequence ID No. 1). The resulting vector was digested with BamHI and HindIII and the ~2.2-kb fragment containing nucleotides 1-831 of Sequence ID No. 1, plus nucleotides 1-63 of Sequence ID No. 3 and nucleotides 832-2136 of Sequence ID No. 1 was isolated and ligated to BamHI/HindIII-digested NMDAR1 to generate NMDAR1-I63.

NMDAR1-I63 contains 261 base pairs of 5' untranslated sequence (nucleotides 1-261 in Sequence ID No. 1), the complete coding sequence for the NMDAR1-I63 variant NMDAR1 subunit (nucleotides 262-831 of Sequence ID No. 1, plus nucleotides 1-63 of Sequence ID No. 3 and nucleotides 832-3078 of Sequence ID No. 1) and 1220 nucleotides of 3' untranslated sequence (nucleotides 3079-4298 of Sequence ID No. 1). Thus, NMDAR1-I63 differs from NMDAR1 in that it contains 63 nucleotides that are not present in NMDAR1 (nucleotides 1-63 of Sequence ID No. 3), located between nucleotides 831 and 832 of Sequence ID No. 1. The NMDAR1-I63 subunit variant-encoding sequence is operatively linked to the regulatory elements in pcDNA1 for expression in mammalian cells.

F. NMDAR1-I63-Δ204-Δ363

Full-length construct NMDAR1-I63-Δ204-Δ363 was prepared by replacing the 2861 nucleotide fragment from construct NMDAR1-I63-Δ204 (ie, nucleotides 1438-4298  
 5 Sequence ID. No. 1) with the *KpnI*-*XbaI* (polylinker site) fragment of NMDAR1-Δ363 (ie, nucleotides 1438-2961 and 3325-4298 of Sequence ID No. 1) as depicted in Figure 2. The NMDAR1-I63-Δ204 was completely digested with *XbaI* then  
 10 partially digested with *KpnI* due to the presence of two additional *KpnI* sites in the vector sequence. The resulting 5' NMDAR1-I63-Δ204 fragment, which includes the pcDNA1 vector sequences, was ligated with the 3' *KpnI*-*XbaI* fragment from NMDAR1-Δ363 to generate NMDAR1-I63-Δ204-Δ363.

In summary, construct NMDAR1-I63-Δ204-Δ363  
 15 contains 261 base pairs of 5' untranslated sequence (nucleotides 1-261 in Sequence ID No. 1), the complete coding sequence for the NMDAR1-I63-Δ204-Δ363 variant NMDAR1A subunit (nucleotides 262-831 of Sequence ID No. 1, plus nucleotides 1-63 of Sequence ID No. 3, plus  
 20 nucleotides 832-984, 1189-2961 and 3325-3393 of Sequence ID No. 1) as well as 905 base pairs of 3' untranslated sequence (nucleotides 3394-4298 of Sequence ID. No. 1). Thus, NMDAR1-I63-Δ204-Δ363 differs from NMDAR1A in that it contains 63 nucleotides that are not present in NMDAR1A  
 25 (nucleotides 1-63 of Sequence ID No. 3) located between nucleotides 831 and 832 of Sequence ID No. 1. Further, NMDAR1-I63-Δ204-Δ363 lacks 204 nucleotides that are present in NMDAR1A (nucleotides 985-1188 of Sequence ID No. 1) and 363 nucleotides that are present in NMDAR1A (nucleotides  
 30 2962-3324 of Sequence ID No. 1) that comprise the last 117 nucleotides of the coding sequence and the first 246 nucleotides of the 3' untranslated sequence of NMDAR1A. The NMDAR1-I63-Δ204-Δ363 subunit variant encoding sequence is operatively linked to the regulatory elements in pcDNA1  
 35 for expression in mammalian cells.

204070" 2E68E00F

G. NMDAR1-I63-Δ204-Δ1087

Full-length construct NMDAR1-I63-Δ204-Δ1087 was prepared by replacing the 2861 nucleotide fragment from construct NMDAR1-I63-Δ204 (ie, nucleotides 1438-4298  
5 Sequence ID. N. 1) with the KpnI-XbaI (polylinker site) fragment of NMDAR1-Δ1087 (ie, nucleotides 1438-2961 and 4049-4298 of Sequence ID No. 1) as depicted in Figure 2. The NMDAR1-I63-Δ204 was completely digested with XbaI then  
10 partially digested with KpnI due to the presence of two additional KpnI sites in the vector sequence. The resulting 5' NMDAR1-I63-Δ204 fragment, which includes the pCDNAI vector sequences, was ligated with the 3' KpnI-XbaI fragment from NMDAR1-Δ1087 to generate NMDAR1-I63-Δ204-Δ1087.

15 In summary, construct NMDAR1-I63-Δ204-Δ1087 contains 261 base pairs of 5' untranslated sequence (nucleotides 1-261 in Sequence ID No. 1), the complete coding sequence for the NMDAR1-I63-Δ204-Δ363 variant NMDAR1A subunit (nucleotides 262-831 of Sequence ID No. 1,  
20 plus nucleotides 1-63 of Sequence ID No. 3, plus nucleotides 832-984, 1189-2961 and 4280-4298 of Sequence ID No. 1) as well as 19 base pairs of 3' untranslated sequence (nucleotides 4280-4298 of Sequence ID. No. 1). Thus, NMDAR1-I63-Δ204-Δ1087 differs from NMDAR1A in that it  
25 contains 63 nucleotides that are not present in NMDAR1A (nucleotides 1-63 of Sequence ID No. 3) located between nucleotides 831 and 832 of Sequence ID No. 1. Further, NMDAR1-I63-Δ204-Δ1087 lacks 204 nucleotides that are present in NMDAR1A (nucleotides 985-1188 of Sequence ID No.  
30 1) and 1087 nucleotides that are present in NMDAR1A (nucleotides 2962-4048 of Sequence ID No. 1) that comprise the last 117 nucleotides of the coding sequence and the first 970 nucleotides of the 3' untranslated sequence of NMDAR1A. The NMDAR1-I63-Δ204-Δ1087 subunit variant

204070 2668007



encoding sequence is operatively linked to the regulatory elements in pcDNA1 for expression in mammalian cells.

H. Additional Constructs Containing Full-Length cDNAs Encoding Variants of the NMDAR1 Subunit

5 Additional full-length cDNAs encoding further possible NMDAR1 variants can be constructed using methods similar to those described in Examples 2A-G above. Specifically, the following constructs can be prepared by ligating portions of clones NMDA11, NMDA10, NMDA7 and NMDA3  
10 as depicted in Figure 2:

	NMDAR1- $\Delta$ 204	(Sequence ID No. 1J)
	NMDAR1- $\Delta$ 204- $\Delta$ 363	(Sequence ID No. 1K)
	NMDAR1-I63- $\Delta$ 363	(Sequence ID No. 1M)
	NMDAR1-I63- $\Delta$ 1087	(Sequence ID No. 1N)
15	NMDAR1- $\Delta$ 204- $\Delta$ 1087	(Sequence ID No. 1L)

The full-length cDNAs can also be incorporated into mammalian expression vectors such as pcDNA1, as described in Examples 2A-G.

20 Several methods can be employed to determine which NMDAR1 subunit variants are actually expressed in various human tissues. For example, oligonucleotides specific for the nucleotide sequences located 5' and 3' of the insertions and deletions of the NMDAR1 transcripts described herein can be used to prime nucleic acid  
25 amplifications of RNA isolated from various tissues and/or cDNA libraries prepared from various tissues. The presence or absence of amplification products and the sizes of the products indicate which variants are expressed in the tissues. The products can also be characterized more  
30 thoroughly by DNA sequence analysis.

RNase protection assays can also be used to determine which variant transcripts are expressed in various tissues. These assays are a sensitive method for detecting and quantitating an RNA species in a complex mixture of total cellular RNA. A portion of the NMDAR1 subunit variant DNA is labeled and hybridized with cellular RNA. If complementary mRNA is present in the cellular RNA, a DNA-RNA hybrid results. The RNA sample is then treated with RNase, which degrades single-stranded RNA. Any RNA-DNA hybrids are protected from RNase degradation and can be visualized by gel electrophoresis and autoradiography.

Further information on possible splice variants of the NMDAR1 primary transcript can be obtained by isolation of genomic clones containing NMDAR1 subunit-encoding sequences (for example, by hybridization to the human NMDAR1 subunit cDNAs disclosed herein) and subsequent characterization of the resulting clones.

### Example 3

#### Isolation of DNA Encoding Human NMDA Receptor

##### NMDAR2C Subunits

Degenerate oligonucleotides were synthesized based on two conserved regions of rat NMDAR2A, NMDAR2B and NMDAR2C DNAs that encode the putative first and fourth transmembrane domains. In rat NMDAR2A DNA, these regions are encoded by nucleotides 1669-1692 (oligo SE74) and 2437-2465 (oligo SE75), respectively. [see Monyer et al. (1992) *Science* 256:1217-1221]. These oligonucleotides were used to prime nucleic acid amplification of cDNAs prepared from RNA isolated from human hippocampus, cerebellum, and orbitofrontal tissue. Two products, a 795-bp and a 640-bp fragment, were detected when the reaction mixture was analyzed by gel electrophoresis and ethidium bromide staining. The 795-bp fragment amplified from the cerebellum cDNA was subcloned into PCR1000 (Invitrogen, San

Diego, CA) and characterized by DNA sequence analysis, which revealed that it is ~86% similar to the rat NMDAR2A DNA sequence, ~78% similar to the rat NMDAR2B DNA sequence, and ~74% similar to the rat NMDAR2C DNA sequence. Thus, this plasmid was named pcrNMDAR2A.

The 795-bp insert from pcrNMDAR2A was used to screen  $1 \times 10^6$  recombinants of a human hippocampus cDNA library (prepared by using random primers to synthesize cDNAs from hippocampus tissue and selecting fragments >2.0 kb for insertion into  $\lambda$ gt10 vectors) and a human cerebellum cDNA library (random-primed library size-selected for fragments >2.8 kb in  $\lambda$ gt10). Hybridization was performed in 5X SSPE, 5X Denhart's solution, 50% deionized formamide, 0.2% SDS, 200  $\mu$ g/ml sonicated, denatured herring sperm DNA at 42°C. Washes were performed in 1X SSPE, 0.2% SDS at 55°C. The probe hybridized to 11 plaques from the hippocampus library and 8 plaques from the cerebellum library.

DNA sequence analysis and/or restriction enzyme mapping of 15 of the hybridizing plaques that were purified surprisingly revealed that they were more similar to rat NMDAR2C DNA than to rat NMDAR2A DNA. All of the clones were partial cDNAs (i.e., they lacked a translation initiation and/or termination codon) and were designated as NMDAR2C cDNAs. Comparison of the clones revealed that the human NMDAR2C subunit transcript is differentially processed.

Clones NMDA26, NMDA24, NMDA22 and NMDA21 (see Figure 4) represent four basic clones that were identified, all of which are believed to be splice variants. Clone NMDA26 (Sequence ID No. 5D) is used as a reference to which the other variants can be compared. Clone NMDA24 (Sequence ID No. 5C) contains a 24-bp sequence (see Sequence ID No. 7) that is not present in NMDA26. Clone NMDA22 (Sequence

ID No. 5B) lacks 15 bp that are present in NMDA26, and clone NMDA21 (Sequence ID No. 5A) lacks 51 bp that are present in NMDA26. Clones NMDA22 and NMDA24 both contain an 11-bp sequence (Sequence ID No. 9) that is not present in NMDA26 (between nucleotides 1116-1117 of Sequence ID No. 5). Introduction of this sequence into these clones (between nucleotides 1116-1117 of Sequence ID No. 5) disrupts the reading frame of the transcript and introduces a premature translation termination (i.e., STOP) codon into the transcript.

Clones NMDA26 and NMDA27 (see Figure 4) are partial NMDAR2C cDNAs that contain 5' untranslated sequence, a translation initiation codon and some of the coding sequence. Clone NMDA26 contains 188 base pairs of 5' untranslated sequence whereas clone NMDA27 contains ~1.1 kb of 5' untranslated sequence. The sequences of the 5' untranslated regions of these two clones are identical for the first 15 nucleotides proceeding 5' of the translation initiation codon. However, beginning with the 16th nucleotide 5' of the translation initiation codon, the sequences of the two clones diverge (compare nucleotides 116-191 of Sequence ID No. 5 to nucleotides 1 - 74 of Sequence ID No. 12).

#### Example 4

##### Preparation of Full-length NMDAR2C Subunit cDNA Constructs

Portions of the partial NMDAR2C clones can be ligated in a variety of ways to generate constructs encoding full-length NMDAR2C subunit variants. The 5' end of each NMDAR2C cDNA can be contributed by NMDA26, whereas the 3' ends of the constructs are contributed by various combinations of clones NMDA21, NMDA22, and NMDA24. Figure 5 depicts full-length NMDAR2C constructs and indicates the portions of the different clones that contribute to each construct.

For example, full-length constructs can be prepared using methods such as those described in Example 2 for preparing NMDAR1 constructs. Thus, clone inserts are transferred into a vector (e.g., pCDNA1) for ease of manipulation and then desired portions of the cDNAs are isolated by restriction enzyme digestion of the vectors. This can require several steps and/or partial digests if, for example, there are no unique restriction enzyme sites surrounding the desired portions of the cDNAs. The desired cDNA fragments are then ligated and incorporated into an expression plasmid such as pCDNA1 or pCMV-T7-2.

Plasmid pCMV-T7-2 (see Figure 6) is a pUC19-based vector that contains a cytomegalovirus (CMV) promoter/enhancer, SV40 splice donor/splice acceptor sites located immediately downstream of the promoter, a T7 bacteriophage RNA polymerase promoter positioned downstream of the SV40 splice sites, an SV40 polyadenylation signal downstream of the T7 promoter, and a polylinker between the T7 promoter and the polyadenylation signal. This vector thus contains all the regulatory elements required for expression of heterologous DNA in a mammalian host cell, wherein the heterologous DNA has been incorporated into the vector at the polylinker. In addition, because the T7 promoter is located just upstream of the polylinker, this plasmid can be used for synthesis of *in vitro* transcripts of heterologous DNA that has been subcloned into the vector at the polylinker. Plasmid pCMV-T7-3, also depicted in Figure 6, is identical to pCMV-T7-2 except that the order of the restriction enzyme sites in the polylinker is reversed. This plasmid can also be used for heterologous expression of NMDAR subunit DNA.

Construct pCDNA1-26-NotI-24-5'UT contains 188 base pairs of 5' untranslated sequence (nucleotides 1-188 of Sequence ID No. 5), the complete coding sequence of the first variant of the human NMDAR2C subunit (nucleotides

189-3899 of Sequence ID No. 5) and ~440 base pairs of 3' untranslated sequence (nucleotides 3900-4340 of Sequence ID No. 5). The NMDAR2C cDNA is contained within the polylinker of expression vector pcDNA1 for expression.

- 5 Construct pCMV-26-NotI-24 (Sequence ID No. 5) contains 49 base pairs of 5' untranslated sequence (nucleotides 140-188 of Sequence ID No. 5), the complete coding sequence of a first variant of the human NMDAR2C subunit (nucleotides 189-3899 of Sequence ID No. 5) and  
10 ~440 base pairs of 3' untranslated sequence (nucleotides 3900-4340 of Sequence ID No. 5). The NMDAR2C cDNA is contained within the polylinker of expression vector pCMV-T7-2 for expression.

- Construct pCMV-26-ScaI-24 (Sequence ID No. 5E) is  
15 identical to pCMV-26-NotI-24, except it contains 24-base pairs (Sequence ID No. 7) inserted between nucleotides 2350 and 2351 of Sequence ID No. 5.

- Construct pCMV-26-ScaI-22 (Sequence ID No. 5F) is identical to pCMV-26-NotI-24, except that it lacks 15-base  
20 pairs (nucleotides 1960-1974 of Sequence ID No. 5).

Construct pCMV-26-ScaI-21-NotI-24 (Sequence ID No. 5G) is identical to pCMV-26-NotI-24, except that it lacks 51-base pairs (nucleotides 2351-2401 of Sequence ID No. 5).

- 25 Construct NMDAR2C- $\Delta$ 15-I24 (Sequence ID No. 5H) is identical to pCMV-26-NotI-24, except that it lacks 15-base pairs (i.e., nucleotides 1960-1974 of Sequence ID No. 5) and includes a 24-base pair sequence (i.e., Sequence ID No. 7; inserted between nucleotides 2350 and 2351 of Sequence  
30 ID No. 5).

204070 268807 103893 040402

Construct NMDAR2C- $\Delta$ 15- $\Delta$ 51 (Sequence ID No. 5I) is identical to pCMV-26-NotI-24, except that it lacks 15-base pairs (i.e., nucleotides 1960-1974 of Sequence ID No. 5) and 51-base pairs (i.e., nucleotides 2351-2401 of Sequence ID No. 5).

Additional full-length NMDAR2C constructs can readily be prepared as described herein. For example, 5' untranslated sequence obtained from NMDA27 (instead of NMDA26) can be employed, and the 3' ends of the constructs can be contributed by various combinations of clones NMDA21, NMDA22, and NMDA24.

Several methods (e.g., nucleic acid amplification, RNase protection assays, etc.), as described in Example 2, can be employed to determine which NMDAR2C subunit variants are actually expressed in various human tissues.

Human NMDAR2C has 83.5% GC nucleotide content between nucleotides 2957 and 3166. To potentially enhance NMDAR2D subunit expression, the GC content in this region can be reduced while maintaining the native amino acid sequence. Synthetic DNAs can be made by oligonucleotide primer extension across this region. Four oligonucleotides, SE343 (Sequence ID No. 17), SE344 (Sequence ID No. 18), SE345 (Sequence ID No. 19), and SE346 (Sequence ID No. 20) were synthesized. These primers maintain the amino acid sequence of the human NMDAR2D receptor and some restriction sites, but lower the overall GC content of this region to 53.4%. The criteria for the modification of bases were: 1) to not have more than 4 guanine nucleotides in a row if at all possible, 2) to maintain the restriction cutting sites for NotI (nucleotides 2962 - 2969 of Sequence ID No. 5), AvaII (nucleotides 3069 - 3073 Sequence ID No. 5), and AatII (nucleotides 3156 - 3161 of Sequence ID No. 5), 3) to

204070 268800

reduce the secondary structure of the oligonucleotides as much as possible, 4) to not introduce any additional NotI, AvaII or AatII restriction sites into the sequence and 5) to have the basepair overlap between oligonucleotide pairs, {SE343 and SE344} or {SE345 and SE346} have a proposed melting temperature between 62-66°C. The oligonucleotide pair SE343 and SE344 have complementary sequence from nucleotides 51 - 71 of Sequence ID Nos. 17 and 18. The oligonucleotide pair SE345 and SE346 have complementary sequence from nucleotides 42 - 61 of Sequence ID No. 19 and nucleotides 43 - 62 of Sequence ID No. 20, respectively.

The primer pairs, {SE343 and SE344} and {SE345 and SE346}, are combined in a standard PCR reaction mixture, which contains 50 pmoles of each oligonucleotide, and are amplified according to the following PCR protocol:

Annealing temperature of 55°C for 1 min, extension temperature of 72°C for 2 min and melting temperature, 96°C for 30 seconds for 30 cycles.

The resulting 121 bp PCR product from the primer pair SE343-SE344 is digested with NotI and AvaI, and the resulting 103 bp PCR product from the primer pair SE345-SE346 is digested with AvaI and AatII. These fragments are ligated into pCMV-NMDAR2C-26-NotI-24, which has been partially digested with both NotI and AatII due to the presence of additional NotI and/or AatII restriction sites in the vector sequence, to form pCMV-26-NotI-24-GCMOD. This construct, pCMV-26-NotI-24-GCMOD, contains nucleotides 140-2965 of Sequence ID No. 5, followed by the 195 nucleotides set forth in Sequence ID No. 21, and then nucleotides 3161 to 4340 of Sequence ID. No. 5.



Example 5Isolation of DNA Encoding Human NMDA Receptor  
NMDAR2A Subunits

Two human cDNA libraries were prepared using  
5 different oligonucleotides (random and specific primers) to  
prime cDNA synthesis from RNA isolated from cerebellum  
tissue. The specific primer used for first-strand  
synthesis was SE162, nucleotides 904 to 929 of Sequence ID  
No. 10. cDNAs synthesized by random priming that ranged in  
10 size from 1.0-2.8 kb, and cDNAs synthesized by specific  
priming that ranged in size from 0.6-1.1 kb, were isolated  
and inserted into the  $\lambda$ gt10 phage vector to generate the  
two libraries.

The random-primed library ( $3 \times 10^6$  recombinants)  
15 was screened for hybridization to the 795-base pair insert  
from pcrNMDAR2A (see Example 3) in 5X SSPE, 5X Denhart's  
solution, 50% deionized formamide, 0.2% SDS, 200  $\mu$ g/ml  
sonicated, denatured herring sperm DNA at 42°C. Washes  
were performed in 1X SSPE, 0.2% SDS at 55°C. The probe  
20 hybridized to 11 plaques.

The specifically-primed library ( $6 \times 10^5$   
recombinants) was screened for hybridization to  
oligonucleotide SE177 (nucleotides 859 to 884 of Sequence  
ID No. 10) in 6X SSPE, 5X Denhart's solution, 10% deionized  
25 formamide, 0.2% SDS, 200  $\mu$ g/ml sonicated, denatured herring  
sperm DNA at 42°C. Washes were performed in 1X SSPE, 0.2%  
SDS at 50°C. The probe hybridized to 2 plaques.

Nine of the hybridizing plaques were purified and  
the inserts were characterized by restriction enzyme  
30 mapping and DNA sequence analysis. All clones contained  
partial cDNAs. Two of the clones, NMDA53 and NMDA54,  
contain the translation initiation codon and 320 base pairs  
and 88 base pairs, respectively, of 5' untranslated

sequence. The sequences of four other clones, NMDA47, NMDA49, NMDAR50 and NMDA51, along with those of NMDA53 and NMDA54, overlap to comprise ~70% of the human NMDAR2A subunit coding sequence (see nucleotides 1 - 3084 of Sequence ID No. 10).

To obtain clones containing the remaining ~1300 base pairs of 3' sequence needed to complete the NMDAR2A coding sequence,  $6.6 \times 10^6$  recombinants of an additional human cDNA library (an amplified randomly primed cerebellum cDNA library with inserts ranging from 1.0 - 2.8 kb in length) were screened for hybridization to an oligonucleotide corresponding to the 3' end of clone NMDA51 (oligo SE171; nucleotide 3454 to 3479 of Sequence ID No. 10) using the same conditions as used for screening the specifically primed cerebellum cDNA library as described above. Four hybridizing plaques were purified and the inserts were characterized by DNA sequence analysis to determine if they contain the 3' end of the coding sequence and a translation termination codon. Two of the clones (NMDA57 and NMDA58, which were determined to be identical), contain a translation termination codon, as determined by DNA sequence analysis. Phage lysate containing clone NMDA57 were deposited under the provisions of the Budapest Treaty with the American Type Culture Collection (ATCC) on April 13, 1993, and assigned Accession No. 75442.

#### Example 6

##### Preparation of Full-length NMDAR2A Subunit cDNA Constructs

Two separate constructs encoding a full-length NMDAR2A subunit (pCMV-hNMDAR2A-1(53) and pCMV-hNMDAR2A-2(54)) were prepared by ligating portions of the following partial NMDAR2A clones: NMDAR47, NMDAR50, NMDAR58 and either NMDAR53 or NMDAR54 (NMDAR53 and NMDAR54 differ only in the amount of 5' untranslated sequence contained in the clones. The inserts of clones NMDA47,

5

10

15

20

25

30

To generate two constructs containing identical NMDAR2A coding sequences but differing amounts of 5' untranslated sequence, pNMDA53 and pNMDA54 were digested with *ScaI/EcoRI* to liberate fragments containing a 3' portion of the  $\beta$ -lactamase gene and nucleotides 1-854 and 225-854 of Sequence ID No. 10, respectively. pNMDA47+50+58 was digested with *ScaI/EcoRI* (partial) and the 3954-bp fragment containing a 5' portion of the  $\beta$ -lactamase gene and nucleotides 855-4808 of Sequence ID No. 10 was separately ligated with the *ScaI/EcoRI* fragments of pNMDA53 and pNMDA54 to generate pCMV-hNMDAR2A-1(53) and pCMV-hNMDAR2A-2(54), respectively. These two constructs are identical except for the amount of 5' untranslated sequence contained in each. Both contain a full-length NMDAR2A-encoding sequence (nucleotides 311-4705 of Sequence ID No. 10) and 103 nucleotides of 3' untranslated sequence (nucleotides 4706-4808 of Sequence ID No. 10). pCMV-hNMDAR2A-1(53) contains 310 nucleotides of 5' untranslated sequence (nucleotides 1-310 of Sequence ID No. 10), whereas pCMV-hNMDAR2A-2(54) contains 87 nt of 5' untranslated sequence (nucleotides 224-310 of Sequence ID No. 10). The NMDAR2A cDNA is operatively linked to the regulator elements of pCMV-T7-2 for expression in mammalian host cells.

There is no unique restriction site 3' of the NMDAR2A-specific DNA in pCMV-hNMDAR2A-1(53) that can be used to linearize the plasmid in order to prepare *in vitro* transcripts for injection into *Xenopus* oocytes. To make a construct that has a unique 3' restriction site (pCMV-hNMDAR2A-3(53)), essentially the entire NMDAR2A-specific DNA of pCMV-hNMDAR2A-1(53) was transferred into vector pCMV-T7-3 as follows. pCMV-NMDAR2A-1(53) was digested with *NotI* and the ~4.4-kb fragment was isolated and ligated with *NotI*-digested pCMV-T7-3 to generate pCMV-hNMDAR2A-3(53).

Example 7Isolation of DNA Encoding Human NMDA Receptor  
NMDAR2B Subunits

A human fetal brain λZAP cDNA library ( $1 \times 10^6$   
 5 recombinants; Stratagene, La Jolla, CA) was screened for  
 hybridization to a DNA fragment containing the entire rat  
 NMDAR2B subunit coding sequence (see Monyer et al. (1992)  
 Science 256:1217-1221). Hybridization was conducted in 50%  
 deionized formamide, 5X Denhart's solution, 5X SSPE, 200  
 10  $\mu\text{g/ml}$  sonicated, denatured herring sperm DNA and 0.2% SDS  
 at 42°C. Washes were performed in 0.5X SSPE, 0.2% SDS at  
 65°C. One of the hybridizing clones excised from the human  
 fetal brain library, NMDA81, containing a 5,435 bp insert  
 and translation initiation and termination codons, encodes  
 15 a full-length NMDAR2B subunit. This excised plasmid, which  
 is in the pBluescript vector, was called pBS-hNMDAR2B.

NMDA81 was digested with *EcoRI/EcoRV* and the  
 ~5.5-kbp fragment was isolated and ligated to *EcoRI/EcoRV*-  
 digested pCMV-T7-3. The resulting construct, pCMVPL3-  
 20 hNMDAR2B, contains the NMDAR2B coding sequence (nucleotides  
 210-4664 of Sequence ID No. 13), as well as 209 nucleotides  
 of 5' untranslated sequence (nucleotides 1-209 of Sequence  
 ID No. 13) and 339 nucleotides of 3' untranslated sequence  
 (nucleotides 4665-5003 of Sequence ID No. 13). The  
 25 NMDAR2B-encoding DNA in this construct is operatively  
 linked to regulatory elements in pCMV-T7-3 for expression  
 in mammalian host cells.

Example 8Isolation of DNA Encoding Human NMDA  
Receptor NMDAR2D subunits

30

A human fetal brain cDNA library ( $1 \times 10^6$   
 recombinants; Stratagene, La Jolla, CA) was screened by  
 subtraction screening methods for DNA encoding a human

204070 2E68E00T

NMDAR2D receptor subunit. In this method, plaques were selected on the basis of weak or no hybridization to DNAs encoding human NMDAR2A, NMDAR2B and NMDAR2C subunits.

Initially, the library was screened for hybridization to pcrNMDAR2A (see Example 3) under low-stringency conditions (30% deionized formamide, 5X Denhart's solution, 5X SSPE, 200 ng/ml sonicated herring sperm DNA, 0.2% SDS at 42°C). Washes were also performed using low-stringency conditions (2X SSPE, 0.2% SDS, 50°C). The filters were stripped, then screened for hybridization to the pcrNMDAR2A fragment and to an ~1200 bp PstI fragment of DNA encoding a human NMDAR2B subunit (see Example 7) and an ~950 bp AccI fragment of DNA encoding a human NMDAR2C subunit (see Example 3). These fragments contain DNA encoding all of the putative transmembrane domains of the subunits. Hybridization was performed under high-stringency conditions (50% deionized formamide, 5X Denhart's solution, 5X SSPE, 200 ng/ml sonicated herring sperm DNA, 0.2% SDS at 42°C) as were washes (0.1X SSPE, 0.1% SDS, 65°C).

Eighteen of the plaques that hybridized weakly to pcrNMDAR2A in the initial low stringency screening of the library hybridized only weakly or not at all to portions of DNA encoding human NMDAR2A, NMDAR2B and NMDAR2C subunits in the high stringency screening. The plaques were purified, and the insert fragments were characterized by DNA sequence analysis. One of the inserts, NMDA96, corresponds to the 3' half of the human NMDAR2D subunit gene coding sequence. The sequence of this clone is provided in Sequence ID No. 15.

To obtain clones containing the remaining ~2000 bp of 5' sequence needed to complete the NMDAR2D subunit coding sequence, the human fetal brain cDNA library was screened for hybridization to an ~831 bp SmaI fragment of

the clone containing the 3' half of the NMDAR2D coding sequence under high stringency hybridization and washing with 0.5X SSPE, 0.2% SDS at 65°C. Nine hybridizing plaques were purified and analyzed by DNA sequencing, which  
 5 revealed that none of the plaques contain DNA encoding a translation initiation codon and extending 3' to at least the 5' end of the clone containing the 3' half of the NMDAR2D coding sequence.

A human cDNA library was prepared using a  
 10 specific oligonucleotide, SE296, to prime cDNA synthesis from RNA isolated from human fetal brain. The specific primer used for first-strand synthesis was SE296 (nucleotides 2920-2949 of Sequence ID No. 15). cDNAs synthesized by specific priming that were greater than 2.2  
 15 kb in size were isolated and inserted into the  $\lambda$ ZAPII phage vector to generate the library.

The specifically primed library ( $1 \times 10^6$  recombinants) was screened for hybridization to the 831 bp *Sma*I fragment from NMDAR2D (nucleotides 2435-3265 of  
 20 Sequence ID No. 15) in 5X SSPE, 5X Denhart's solution, 50% deionized formamide, 0.2% SDS, 200  $\mu$ g/ml sonicated, denatured herring sperm DNA at 42°C. Washes were performed in 0.1X SSPE, 0.2% SDS at 65°C. One probe hybridized to 11 plaques.

25 Eleven of the hybridizing plaques were purified, and the inserts characterized by restriction enzyme mapping and DNA sequence analysis. Six of the clones (NMDA111, NMDA112, NMDA115, NMDA116, NMDA119 and NMDA121) contain the translation initiation codon and varying amounts of 5'  
 30 untranslated sequence.

The sequences of these clones overlap with NMDA96 to constitute 100% of the human NMDAR2D subunit coding sequence (see nucleotides 485-4495 of Sequence ID No. 15).

10038937.010102

The full-length hNMDAR2D construct was prepared using NMDA115 and NMDA96 cDNAs. NMDA115 and NMDA96 cDNAs are already in the pBlueScript vector, however the NMDA115 cDNA is in the sense orientation from the T7 promoter, while the NMDA96 cDNA is in the antisense orientation. For ease of subcloning the full-length construct, the NMDA96 cDNA was cloned into the sense orientation by digesting NMDA96 with *EcoRI* and screening the resulting clones for orientation (NMDAR96-T7). Within the complete human NMDAR2D sequence, there is a unique *HindIII* at nucleotides 2804 that was used to clone NMDA115 together with NMDA96. However, there is an additional *HindIII* site in the pBS polylinker at the 5' end of the NMDA115 cDNA. Therefore NMDA115 was fully digested with *SpeI*, a 3' polylinker site, and partially digested with *HindIII*. The resulting ~5.6 kb *SpeI-HindIII* fragment from pNMDA115 (pBS vector plus nucleotides 397-2804 of Sequence ID No. 15) was ligated with the 1.7 kb *HindIII-SpeI* fragment (nucleotides 2805-4651 of Sequence ID No. 15) from NMDA96-T7 to form pBS-hNMDAR2D. *In vitro* transcripts were prepared for co-injection into *Xenopus* oocytes to test for alteration of NMDAR1A currents.

The complete NMDAR2D insert is then transferred into the pMTV-T7+ mammalian expression vector as a ~4.7 kb *EcoRV/SpeI* fragment. The *EcoRV* and *SpeI* restriction sites are in the multiple cloning region of the pBluscript vector.

In summary, construct NMDAR2D contains 88 base pairs of 5' untranslated sequence (nucleotides 397-484 in Sequence ID No. 15), the complete coding sequence for the NMDAR2D subunit (nucleotides 484-4495 of Sequence ID No. 15) as well as 200 base pairs of 3' untranslated sequence (nucleotides 4496-4695 of Sequence ID No. 15). The NMDAR2D subunit encoding sequence is operatively linked to the



regulatory elements in pMMTV-T7 for expression in mammalian cells.

### Example 9

#### Expression of Recombinant Human NMDA

5

#### Receptor Subunits on Oocytes

*Xenopus* oocytes were injected with in vitro transcripts prepared from constructs containing DNA encoding human NMDA receptor NMDAR1 and NMDAR2 subunits. Electrophysiological measurements of the oocyte transmembrane currents were made using the two-electrode voltage clamp technique (see e.g., Stuhmer (1992) *Meth. Enzymol.* 207:319-339).

#### A. Preparation of In Vitro Transcripts

Recombinant capped transcripts of NMDA receptor subunit cDNAs contained in constructs NMDAR1A, NMDAR1-I63, NMDAR1-I63- $\Delta$ 204, NMDAR1- $\Delta$ 1087, NMDAR1- $\Delta$ 363, and pCMV-26-NotI-24 were synthesized from linearized plasmids using the mCAP RNA Capping Kit (Cat. #200350, Stratagene, Inc., La Jolla, CA). For experiments in which NMDAR2A or NMDAR2B and NMDAR1 or NMDAR1-I63 transcripts were co-injected into *Xenopus* oocytes, the transcripts were synthesized from linearized constructs NMDAR1A, NMDAR1-I63, pCMV-hNMDAR2A-3(53), pCMV-26-NotI-24 and pBS-hNMDAR2B using mMessage mMachine (Ambion, catalog #1344, Austin, TX). The mass of each synthesized transcript was determined by UV absorbance and the integrity of each transcript was determined by electrophoresis through an agarose gel.

#### B. Electrophysiology

*Xenopus* oocytes were injected with 12.5-50 ng of one or more NMDA receptor subunit transcripts per oocyte. The preparation and injection of oocytes were carried out

204070-2668E001

as described by Dascal [(1987) *Crit. Rev. Biochem.* 22:317-387]. Two-to-six days following mRNA injection, the oocytes were examined using the two-electrode voltage clamp technique. The cells were bathed in Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.3), and the membrane potential was clamped at -80 to -100 mV. Drugs were applied by pipetting 6.0  $\mu$ l aliquots of drug-containing solution directly into the bath, or by using gravity-feed into a Warner Instruments chamber (volume = 110  $\mu$ l) at a flow rate of 8 ml/min. The data were sampled at 2-5 Hz with a Labmaster data acquisition board in a PC-386 using AXOTAPE version 1.2 (Axon Instruments, Foster City, CA) software. The data were exported to a laser printer or plotted using Sigmaplot version 5.0.

NMDA agonists, i.e., 10-30  $\mu$ M glycine (gly) and 10-100  $\mu$ M glutamate (glu) or 100-1000  $\mu$ M NMDA, were applied to the bath. If a current response was observed, the agonists were washed from the bath and 0.1-1.0 mM MgCl<sub>2</sub> or 1  $\mu$ M MK801 (Research Biochemicals, Inc., Natick, MA) (NMDA receptor antagonists) were applied before a second agonist application in order to determine whether the current was blocked by antagonists. Alternatively, MgCl<sub>2</sub> or MK-801 were applied during agonist-induced current flow. The results of multiple recordings are summarized in Table 1.

Table 1

Electrophysiological Analysis of Oocytes Injected with  
NMDA Receptor Subunit Transcripts

Transcript (ng injected)	No. Oocytes Responding	Agonists	Peak Current Amplitude
NMDAR1A (12.5)	6 of 8 <sup>a</sup>	10 $\mu$ M gly + 10 $\mu$ M glu	3-40 nA <sup>*</sup>
NMDAR1A (12.5)	2 of 2 <sup>a</sup>	10 $\mu$ M gly + 100 $\mu$ M NMDA	3-8 nA
NMDAR1A (12.5)	0 of 9 <sup>a</sup>	10 $\mu$ M gly + 10 $\mu$ M glu	
NMDAR1A (50)	0 of 1 <sup>a</sup>	20 $\mu$ M gly + 20 $\mu$ M glu	
NMDAR1A (40)	4 of 10	10 $\mu$ M gly + 10 $\mu$ M glu	21.3 $\pm$ 20.9 nA <sup>*</sup>
NMDAR1A (40)	1 of 5	10 $\mu$ M gly + 100 $\mu$ M NMDA	24 nA <sup>*</sup>
NMDAR1A (40)	1 of 1	10 $\mu$ M gly + 100 $\mu$ M NMDA	15.4 nA
NMDAR1A (30)	4 of 9	10 $\mu$ M gly + 50 $\mu$ M glu	10.6 $\pm$ 11.7 nA <sup>*</sup>
NMDAR1A (30)	0 of 8	10-20 $\mu$ M gly + 10-100 $\mu$ M glu	
NMDAR1A (30)	1 of 4	20 $\mu$ M gly + 100 $\mu$ M NMDA	10.5 nA
NMDAR1A (25-50)	3 of 3	30 $\mu$ M gly + 100 $\mu$ M glu	3-10 nA
NMDAR1-I63 (12.5)	1 of 5 <sup>a</sup>	10 $\mu$ M gly + 10 $\mu$ M glu	~30 nA <sup>*</sup>
NMDAR1-I63 (50)	0 of 4 <sup>a</sup>	10 $\mu$ M gly + 10 $\mu$ M glu	
NMDAR1-I63 (40)	4 of 5	10 $\mu$ M gly + 10 $\mu$ M glu	13.4 $\pm$ 7.1 nA <sup>+</sup>
NMDAR1-I63 (40)	3 of 3	10 $\mu$ M gly + 20 $\mu$ M glu	17.4 $\pm$ 3.7 nA <sup>*</sup>
NMDAR1-I63 (40)	1 of 1	10 $\mu$ M gly + 100 $\mu$ M glu	28 nA
NMDAR1-I63 (40)	1 of 1	10 $\mu$ M gly + 10 $\mu$ M NMDA	1.4 nA <sup>+</sup>

Transcript (ng injected)	No. Oocytes Responding	Agonists	Peak Current Amplitude
NMDAR1-I63 (25-50)	3 of 3	10 $\mu$ M gly + 100 $\mu$ M glu	3-5 nA
NMDAR1-I63 (40)	7 of 10	10 $\mu$ M gly + 100 $\mu$ M NMDA	8.1 $\pm$ 3.0 nA <sup>+</sup>
NMDAR1-I63 (40)	1 of 2	10 $\mu$ M gly + 1000 $\mu$ M NMDA	16.4 nA <sup>+</sup>
NMDAR1-I63- $\Delta$ 204 (12.5)	0 of 8 <sup>a</sup>	10 $\mu$ M gly + 10 $\mu$ M glu	
NMDAR1-I63- $\Delta$ 204 (50)	1 of 5 <sup>a</sup>	20 $\mu$ M gly + 20 $\mu$ M glu	~50 nA
NMDAR1- $\Delta$ 1087 (50)	3 of 13	10 $\mu$ M gly + 10 $\mu$ M glu	4-11 nA*
NMDAR1A (39) + pCMV-26-NotI-24 (39)	1 of 5	10 $\mu$ M gly + 50 $\mu$ M glu	10 nA
NMDAR1A (30) + pCMV-26-NotI-24 (30)	0 of 7	10 $\mu$ M gly + 20 $\mu$ M glu	
NMDAR1A (32) + pcDNA1-26-NotI-24-5'UT (50)	4 of 5	10 $\mu$ M gly + 10 $\mu$ M glu	15.8 $\pm$ 2.6 nA
NMDAR1A (25-50) + pCMV-hNMDAR2A-3 (53) (25-50)	16 of 29	30 $\mu$ M gly + 100 $\mu$ M glu	40 nA - 3.4 $\mu$ A
NMDAR1-I63 (25-50) + pCMV-hNMDAR2A-3 (53) (25-50)	6 of 11	10 $\mu$ M gly + 100 $\mu$ M glu	10 - 100 nA
NMDAR1A (25) + pBS-hNMDAR2B (25)	4 of 5	30 $\mu$ M gly + 30 $\mu$ M glu	>100 nA
NMDAR1A (50) + pCMV-hNMDAR2A-3 (50) + pCMV-26-NotI-24 (50)	15 of 22	100 $\mu$ M NMDA + 30 $\mu$ M gly -or- 100 $\mu$ M NMDA + 100 $\mu$ M gly	137.7 nA 1340.1 nA

- a Oocytes were unhealthy (i.e., the holding current was large)
- \* The agonist-induced currents in at least 1 cell were blocked by 100  $\mu$ M  $\text{MgCl}_2$ .
- + The agonist-induced currents in at least 1 cell were blocked by 1.0  $\mu$ M MK801.

Analysis of the results shown in Table 1 indicates that, in general, the NMDA agonist-induced currents were blocked by either  $MgCl_2$  or MK801.

Oocytes injected with transcripts (12.5 to 65 ng) of the NMDAR-1 subunit-encoding inserts of constructs NMDAR1A, NMDAR1-I63 or NMDAR1- $\Delta$ 363 were further analyzed to evaluate human NMDA receptor sensitivity to glutamate and NMDA. The two-electrode voltage clamp methods described above were used to measure current in the cells.

To determine glutamate and NMDA sensitivity of the recombinant human NMDA receptors, various concentrations of glutamate (0.1 - 100  $\mu M$ ) or NMDA (3-1000  $\mu M$ ) were applied to the bath (in the presence of 10-30  $\mu M$  glycine) and the current response was recorded. The bath was flushed between agonist applications. Intermediate test applications of 10  $\mu M$  glycine plus 10  $\mu M$  glutamate were included in the experiments to monitor the receptors for run-down (i.e., inactivation of receptors that have been repeatedly activated during prolonged electrophysiological recording). The data were used to generate dose-response curves from which  $EC_{50}$  values for the two agonists were calculated. Glycine sensitivity was determined in the same manner except that various concentrations (0.1-100  $\mu M$ ) of glycine were co-applied with 100  $\mu M$  NMDA.

The  $EC_{50}$  values determined for glutamate stimulation of NMDA receptors expressed in oocytes injected with NMDAR1A, NMDAR1-I63 or NMDAR1- $\Delta$ 363 transcripts were 0.4, 0.6 and 0.5  $\mu M$ , respectively. The  $EC_{50}$  values determined for NMDA stimulation of NMDA receptors expressed in oocytes injected with NMDAR1A, NMDAR1-I63 or NMDAR1- $\Delta$ 363 transcripts were 6.3, 10.9 and 11.9  $\mu M$ , respectively.

There was a marked potentiation of the current magnitude in response to glutamate and glycine in oocytes co-injected with *in vitro* transcripts of pCMV-hNMDAR2A-3(53) and NMDAR1A or NMDAR1-I63 compared to the  
 5 currents recorded in oocytes injected with transcripts of either NMDAR1A or NMDAR1-I63 alone. Similarly, there was a marked potentiation of the current magnitude in response to glutamate and glycine in oocytes co-injected with *in vitro* transcripts of NMDAR1A and pBS-hNMDAR2B compared to  
 10 the currents recorded in oocytes injected with only the NMDAR1A transcript.

To investigate the pharmacological properties of human NMDA receptors generated by coexpression of the human NMDAR1A, NMDAR2A and NMDAR2C subunits, oocytes were co-  
 15 injected with 50 ng each of *in vitro* transcripts prepared from the NMDAR1A, pCMV-hNMDAR2A-3, and pCMV-26-NotI-24 (NMDAR2C) constructs. The sensitivity of the recombinant heteromeric receptors to glycine and NMDA was determined as described above. The  $EC_{50}$  for glycine activation of inward  
 20 currents in these recombinant oocytes was calculated from the dose-response curve to be  $0.87 \pm 0.24 \mu M$  (mean  $\pm$  S.D. of 4 oocytes), which was significantly different than the  $EC_{50}$  calculated for glycine sensitivity of oocytes injected with 50 ng each of *in vitro* transcripts of NMDAR1A and  
 25 pCMV-hNMDAR2A-3 alone ( $1.9 \pm 0.26 \mu M$ ;  $p = 0.0002$ , one-tailed t-test). The sensitivity to NMDA also increased when human NMDAR2C was co-expressed with human NMDAR1A and NMDAR2A subunits. The  $EC_{50}$  for NMDA was shifted from  $30.2 \pm 9.4 \mu M$  for oocytes co-injected with 50 ng each of *in vitro* transcripts of NMDAR1A and pCMV-hNMDAR2A-3 to  $11.9 \pm 5.2 \mu M$  for oocytes co-injected with 50 ng each of *in vitro* transcripts of NMDAR1A, pCMV-hNMDAR2A-3 and pCMV-26-NotI-24  
 30 (mean  $\pm$  S.D. of 4 oocytes).

Example 10Recombinant Expression of Human NMDA Receptor Subunits  
in Mammalian Cells

Mammalian cells, such as human embryonic kidney  
 5 (HEK293) cells can be transiently and/or stably transfected  
 with DNA encoding human NMDA receptor subunits (e.g., DNA  
 encoding an NMDAR1 subunit or DNA encoding an NMDAR1  
 subunit and DNA encoding an NMDAR2 subunit such as pCMV-26-  
 NotI-24, pCMV-hNMDAR2A-3(53) or pCMVPL3-hNMDAR2B).  
 10 Transfectants are analyzed for expression of NMDA receptors  
 using various assays, e.g., northern blot hybridization,  
 electrophysiological recording of cell currents,  $\text{Ca}^{2+}$ -  
 sensitive fluorescent indicator-based assays and [ $^3\text{H}$ ]-MK801  
 binding assays.

15 A. Transient Transfection of HEK Cells

Two transient transfections were performed. In  
 one transfection, HEK 293 cells were transiently  
 transfected with DNA encoding an NMDAR1 (construct NMDAR1A)  
 subunit. In another transfection, HEK 293 cells were  
 20 transiently co-transfected with DNA encoding NMDAR1  
 (construct NMDAR1A) and NMDAR2C (pCMV-26-NotI-24) subunits.  
 In both transfections,  $\sim 2 \times 10^6$  HEK cells were transiently  
 transfected with 19  $\mu\text{g}$  of the indicated plasmid(s)  
 according to standard  $\text{CaPO}_4$  transfection procedures [Wigler  
 25 et al. (1979) *Proc. Natl. Acad. Sci. USA* 76:1373-1376]. In  
 addition, 1  $\mu\text{g}$  of plasmid pCMV $\beta$ gal (Clontech Laboratories,  
 Palo Alto, CA), which contains the *Escherichia coli*  
 $\beta$ -galactosidase gene fused to the CMV promoter, were  
 co-transfected as a reporter gene for monitoring the  
 30 efficiency of transfection. The transfectants were  
 analyzed for  $\beta$ -galactosidase expression by direct staining  
 of the product of a reaction involving  $\beta$ -galactosidase and  
 the X-gal substrate [Jones (1986) *EMBO* 5:3133-3142].  
 Transfectants can also be analyzed for  $\beta$ -galactosidase

204070 2686007



expression by measurement of  $\beta$ -galactosidase activity [Miller (1972) in *Experiments in Molecular Genetics*, pp.352-355, Cold Spring Harbor Press].

The efficiency of these transfections of HEK  
5 cells was typical of standard efficiencies (i.e., ~50%).

B. Stable Transfection of Mammalian Cells

Mammalian cells, such as HEK 293 cells, can be  
stably transfected using the calcium phosphate transfection  
procedure [*Current Protocols in Molecular Biology*, Vol. 1,  
10 Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9  
(1990)]. Ten-cm plates, each containing  $1-2 \times 10^6$  cells,  
are transfected with 10 ml of DNA/calcium phosphate  
precipitate in media containing approximately 19  $\mu$ g of NMDA  
receptor subunit-encoding DNA and 1  $\mu$ g of DNA encoding a  
15 selectable marker, for example, neomycin-resistance gene  
(i.e., pSV2neo). After ~14 days of growth in media  
containing typically 1  $\mu$ g/ml G418, colonies form and are  
individually isolated using cloning cylinders. The  
isolates are then subjected to limiting dilution and  
20 screened to identify those that express NMDA receptors  
using, for example, methods described below.

204070 ZEEB2001

## C. Analysis of Transfectants

### 1. Northern Blot Hybridization Analysis

Total RNA was isolated from  $\sim 1 \times 10^7$  HEK cells co-transfected with NMDAR1 and pCMV-26-NotI-24, and 5-10  $\mu\text{g}$  of RNA was used for northern hybridization analysis. Fragments from human neuronal NMDAR subunit-encoding plasmids were randomly primed and labeled with  $^{32}\text{P}$ -dCTP Klenow incorporation and used as probes. The northern blot hybridization and wash conditions were as follows:

10 hybridization in 5x SSPE, 5X Denhart's solution, 50% formamide, at  $42^\circ\text{C}$  followed by washing in 0.2x SSPE, 0.1% SDS, at  $65^\circ\text{C}$ .

15 Results of these studies revealed the transfectants expressed detectable levels of NMDAR1 and NMDAR2C mRNA of the appropriate size (based on the size of the cDNAs).

### 2. Fluorescent indicator-based assays

20 Activation of ligand-gated NMDA receptors by agonists leads to an influx of cations (both monovalent and divalent), including  $\text{Ca}^{2+}$ , through the receptor channel. Calcium entry into the cell through the channel can in turn induce release of calcium contained in intracellular stores. Monovalent cation entry into the cell through the  
25 channel can also result in an increase in cytoplasmic calcium levels through depolarization of the membrane and subsequent activation of voltage-dependent calcium channels. Therefore, methods of detecting transient increases in intracellular calcium concentration can be  
30 applied to the analysis of functional NMDA receptor expression. One method for measuring intracellular calcium

levels relies on calcium-sensitive fluorescent indicators.

Calcium-sensitive indicators, such as fluo-3 (Catalog No. F-1241, Molecular Probes, Inc., Eugene, OR) are available as acetoxymethyl esters which are membrane permeable. When the acetoxymethyl ester form of the indicator enters a cell, the ester group is removed by cytosolic esterases, thereby trapping the free indicator in the cytosol. Interaction of the free indicator with calcium results in increased fluorescence of the indicator; therefore, an increase in the intracellular  $\text{Ca}^{2+}$  concentration of cells containing the indicator can be expressed directly as an increase in fluorescence. An automated fluorescence detection system for assaying NMDA receptors has been described in commonly assigned pending US Patent Application No. 07/812,254 and corresponding PCT Patent Application No. US92/11090, incorporated by reference herein in their entirety.

Mammalian cells that have been transfected with DNA encoding NMDAR1 or NMDAR1 and NMDAR2 subunits can be analyzed for expression of functional recombinant NMDA receptors using the automated fluorescent indicator-based assay. The assay procedure is as follows.

Untransfected mammalian host cells (or host cells transiently transfected with pCMV-T7-2) and mammalian cells that have been transfected with NMDAR1 ± NMDAR2 subunit DNA are plated in the wells of a 96-well microtiter dish (Nunc Catalog No. 1-6708, available through Alameda Industries, Escondido, CA) that has been precoated with poly-L-lysine at a density of  $2.5 \times 10^5$  cells/well and loaded with fluo-3 by incubation for 2 hours at 20°C in a medium containing 20 μM fluo-3, 0.2% Pluronic F-127 in HBS (125 mM NaCl, 5 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.62 mM  $\text{MgCl}_2$ , 20 mM glucose, 20 mM HEPES, pH 7.4). The cells are then washed with assay

buffer (i.e. HBS). The microtiter dish is then placed into a fluorescence plate reader (e.g., Fluoroskan II, Lab Products International, Ltd., Raleigh, NC) and the basal fluorescence of each well is measured and recorded before  
5 addition of 10  $\mu$ M glycine and 10  $\mu$ M glutamate to the wells. The fluorescence of the wells is monitored repeatedly (75 readings at 0.63-sec intervals) following addition of agonist.

204070 7668001  
10 The fluorescence of the untransfected host cells preferably will not change after addition of glycine and glutamate, i.e., the host cells should not express endogenous excitatory amino acid receptors. The fluorescence of mammalian cells transfected with NMDAR1  $\pm$  NMDAR2 subunit DNA will increase after addition of glycine  
15 and glutamate if a sufficient number of functional NMDA receptors are expressed at the cell surface, and fluorescence readings are taken rapidly.

The resting potential of the membrane of some mammalian host cells may be relatively positive (e.g., -35  
20 mV). Because activation of some NMDA receptors may be significantly reduced at relatively positive potentials, it may be necessary to lower the resting potential of the membrane of cells transfected with human NMDA receptor subunit-encoding DNAs prior to assaying the cells for NMDA  
25 receptor activity using the fluorescent indicator-based assay. This may be accomplished by adding valinomycin (~10  $\mu$ M) to the transfected cells prior to adding NMDA receptor agonists to initiate the assay.

### 3. NMDA Receptor Ligand Binding Assays

30 Mammalian cells transfected with NMDAR1  $\pm$  NMDAR2 subunit DNAs can be analyzed for [ $^3$ H]-MK801 binding. An additional ligand-binding assay for NMDA receptors using

5

i. Buffy coat Homogenate from Rat Cerebral Cortex

25

## 30

In order to prepare membranes from transfected and untransfected mammalian cells, the cells are scraped from the tissue culture plates, and the plates are rinsed with 5 ml of PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.7 mM  $\text{KH}_2\text{PO}_4$ ). The cells are centrifuged at low speed in a table-top centrifuge, and the cell pellet is rinsed with PBS. The cell pellet is resuspended in 20 ml of 10 mM Hepes buffer, pH 7.4, using

5

## 10

15

25

30

of 10  $\mu$ M MK801 is subtracted from the total binding in order to determine the specific binding.

Rat brain cortical buffy coat membranes displayed specific saturable binding of [ $^3$ H]-MK801. In the presence of glycine and glutamate, the ratio of total-to-nonspecific binding (S:N ratio) was 28:1, whereas in the absence of glutamate and glycine the S:N ratio was 5:1. Thus, the binding of MK801 to rat NMDA receptors is potentiated by glutamatergic agonists. Scatchard analysis of [ $^3$ H]-MK801 binding to rat brain membranes indicated that the sensitivity of the assay was 90 fmoles of receptor.

c. [ $^3$ H]-CGP39653 Binding to NMDA Receptors

The binding of [ $^3$ H]-CGP39653 to rat brain membranes is carried out as described by Sills et al. [(1991) *Eur. J. Pharmacol.* 192:19]. The buffy coat membrane pellet is resuspended in 50 volumes of 5 mM Tris-HCl containing 10 mM EDTA, pH 7.7, and incubated for 10 min. at 37°C. The supernatant is centrifuged at 48,000 x g for 10 min. at 4°C. The wash step is repeated once and the pellet is resuspended in 10 ml of 5 mM Tris-HCl containing 10 mM EDTA, pH 7.7. This rat brain membrane suspension is incubated in duplicate or triplicate with 2.0 nM [ $^3$ H]-CGP39653 (New England Nuclear) in a total volume of 0.5 ml assay buffer (5 mM Tris-HCl, pH 7.7) for 60 min at 0°C. Nonspecific binding is determined in the presence of 100  $\mu$ M glutamate. Bound radioactivity is separated from the free by vacuum filtration through GF/C filters which are presoaked for 2-3 hrs in 0.05% polyethylenimine, using the filtration manifold. Unbound radioactivity is removed with two washes of 3 ml each of ice-cold buffer. The filters are dried and transferred to scintillation vials, each containing 10 ml of scintillation fluid. The vials are vortexed, and the radioactivity is measured in a Beckman scintillation counter. The nonspecific binding observed in

the presence of 100  $\mu$ M glutamate is subtracted from the total binding to determine the specific binding.

[<sup>3</sup>H]-CGP39653 binding was first measured as a function of membrane concentration. Specific binding  
5 increased linearly with increasing membrane concentration up to 200  $\mu$ g of protein in the presence of 2 nM [<sup>3</sup>H]-CGP39653.

Saturation analysis of [<sup>3</sup>H]-CGP39653 binding was carried out by incubating 150  $\mu$ g of rat buffy coat  
10 homogenate with increasing concentrations of [<sup>3</sup>H]-CGP39653 for 60 min at 4°C. Scatchard analysis indicated a single class of binding sites with a  $B_{\max}$  value of  $0.69 \pm 0.09$  pmoles/mg and a  $K_d$  value of  $12.3 \pm 0.12$  nM.

While the invention has been described in detail  
15 with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

204070 2E6E00T



Summary of Sequences

Sequence ID No. 1 is a nucleotide sequence encoding a human N-methyl-D-aspartate (NMDA) receptor subunit, NMDAR1A, and the deduced amino acid sequence thereof.

Sequence ID No. 1A is a 3083 nucleotide sequence encoded by clone NMDA10, comprising nucleotides 320 - 3402 of Sequence ID No. 1. Thus, Sequence ID No. 1A differs from Sequence ID No. 1 in that it does not contain the 319 5' nucleotides, nor the 896 3' nucleotides thereof.

Sequence ID No. 1B is a 3155 nucleotide sequence encoded by clone NMDA11, comprising nucleotides 1 - 2961, plus nucleotides 3325 - 3518 of Sequence ID No. 1. Thus, Sequence ID No. 1B differs from Sequence ID No. 1 by the deletion of 363 nucleotides from the 3' portion thereof (i.e., by the deletion of nucleotides 2962 - 3324 of Sequence ID No. 1), and further by the lack of the 781 terminal 3' nucleotides of Sequence ID No. 1.

Sequence ID No. 1C is a 2542 nucleotide sequence encoded by clone NMDA7, comprising nucleotides 556 - 831 of Sequence ID No. 1, plus an additional 63 nucleotides (set forth in Sequence ID No. 3) and nucleotides 832 - 984, 1189 - 2961 and 3325 - 3599 of Sequence ID No. 1. Thus, Sequence ID No. 1C differs from Sequence ID No. 1 in that it does not contain the 555 5'-most nucleotides thereof, it does not contain the 204 nucleotides set forth as nucleotides 985 - 1188 of Sequence ID No. 1, it does not contain the 363 3' nucleotides set forth as nucleotides 2962 - 3324 of Sequence ID No. 1, and it does not contain the 700 3'-most nucleotides of Sequence ID No. 1, while it does contain an additional 63 nucleotides (Sequence ID No. 3) inserted between nucleotides 831 and 832 of Sequence ID No. 1.

Sequence ID No. 1D is a 593 nucleotide sequence encoded by clone NMDA3, comprising nucleotides 2617 - 2961, plus nucleotides 4049 - 4298 of Sequence ID No. 1. Thus, Sequence ID No. 1D differs from Sequence ID No. 1 in that  
 5 it does not contain the 2616 5' nucleotides thereof, and by the deletion of 1087 nucleotides from the 3' portion thereof (i.e., by the deletion of nucleotides 2962 - 4048 of Sequence ID No. 1).

Sequence ID No. 1E is a nucleotide sequence  
 10 encoding human NMDA receptor subunit NMDAR1- $\Delta$ 363, comprising nucleotides 1 - 2961, plus nucleotides 3325 - 4298 of Sequence ID No. 1. Thus, Sequence ID No. 1E differs from Sequence ID No. 1 in that it does not contain the 363 nucleotides set forth as nucleotides 2962 - 3324 of  
 15 Sequence ID No. 1.

Sequence ID No. 1F is a nucleotide sequence encoding human NMDA receptor subunit NMDAR1- $\Delta$ 1087, comprising nucleotides 1 - 2961, plus nucleotides 4049 - 4298 of Sequence ID No. 1. Thus, Sequence ID No. 1F  
 20 differs from Sequence ID No. 1 in that it does not contain the 1087 nucleotides set forth as nucleotides 2962 - 4048 of Sequence ID No. 1.

Sequence ID No. 1G is a nucleotide sequence encoding human NMDA receptor subunit NMDAR1-I63. Sequence  
 25 ID No. 1G is the same as Sequence ID No. 1, further comprising an additional 63 nucleotides (set forth in Sequence ID No. 3) inserted between nucleotides 831 and 832 of Sequence ID No. 1.

Sequence ID No. 1H is a nucleotide sequence  
 30 encoding human NMDA receptor subunit NMDAR1-I63- $\Delta$ 204. Sequence ID No. 1H is the same as Sequence ID No. 1G, except Sequence ID No. 1H does not contain the 204

10038937-040402

nucleotides set forth as nucleotides 985 - 1188 of Sequence ID No. 1.

Sequence ID No. 1I is a nucleotide sequence encoding human NMDA receptor subunit NMDAR1-I63-Δ204-Δ363.  
 5 Sequence ID No. 1I is the same as Sequence ID No. 1H, except Sequence ID No. 1I does not contain the 363 nucleotides set forth as nucleotides 2962 - 3324 of Sequence ID No. 1.

Sequence ID No. 1J is a nucleotide sequence  
 10 encoding human NMDA receptor subunit NMDAR1-Δ204. Sequence ID No. 1J is the same as Sequence ID No. 1, except Sequence ID No. 1J does not contain the 204 nucleotides set forth as nucleotides 985 - 1188 of Sequence ID No. 1.

Sequence ID No. 1K is a nucleotide sequence  
 15 encoding human NMDA receptor subunit NMDAR1-Δ204-Δ363. Sequence ID No. 1K differs from Sequence ID No. 1 in that Sequence ID No. 1K does not contain the 204 nucleotides set forth as nucleotides 985 - 1188 of Sequence ID No. 1, nor the 363 nucleotides set forth as nucleotides 2962 - 3324 of  
 20 Sequence ID No. 1.

Sequence ID No. 1L is a nucleotide sequence encoding human NMDA receptor subunit NMDAR1-Δ204-Δ1087. Sequence ID No. 1L differs from Sequence ID No. 1 in that Sequence ID No. 1L does not contain the 204 nucleotides set  
 25 forth as nucleotides 985 - 1188 of Sequence ID No. 1, nor the 1087 nucleotides set forth as nucleotides 2962 - 4048 of Sequence ID No. 1.

Sequence ID No. 1M is a nucleotide sequence encoding human NMDA receptor subunit NMDAR1-I63-Δ363.  
 30 Sequence ID No. 1M is the same as Sequence ID No. 1G except Sequence ID No. 1M does not contain the 363 nucleotides set forth as nucleotides 2962 - 3324 of Sequence ID No. 1.

204070-2E6E001

Sequence ID No. 1N is a nucleotide sequence encoding human NMDA receptor subunit NMDAR1-I63-Δ1087. Sequence No. 1N is the same as Sequence ID No. 1G except Sequence ID No. 1N does not contain the 1087 nucleotides set forth as nucleotides 2962 - 4048 of Sequence ID No. 1.

Sequence ID No. 1P is a nucleotide sequence encoding human NMDA receptor subunit NMDAR1-I63-Δ204-Δ1087. Sequence ID No. 1P is the same as Sequence ID No. 1H, except Sequence ID No. 1P does not contain the 1087 nucleotides set forth as nucleotides 2962 - 4048 of Sequence ID No. 1.

Sequence ID No. 2 is the amino acid sequence of the NMDA receptor subunit set forth in Sequence ID No. 1.

Sequence ID No. 2A is the amino acid sequence of a portion of an NMDA receptor subunit as encoded by the nucleotide sequence of Sequence ID No. 1A.

Sequence ID No. 2B is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1B.

Sequence ID No. 2C is the amino acid sequence of a portion of an NMDA receptor subunit as encoded by the nucleotide sequence of Sequence ID No. 1C.

Sequence ID No. 2D is the amino acid sequence of a portion of an NMDA receptor subunit as encoded by the nucleotide sequence of Sequence ID No. 1D.

Sequence ID No. 2E is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1E.

204070-265500T

Sequence ID No. 2F is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1F.

Sequence ID No. 2G is the amino acid sequence of  
5 an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1G.

Sequence ID No. 2H is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1H.

Sequence ID No. 2I is the amino acid sequence of  
10 an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1I.

Sequence ID No. 2J is the amino acid sequence of  
15 an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1J.

Sequence ID No. 2K is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1K.

Sequence ID No. 2L is the amino acid sequence of  
20 an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1L.

Sequence ID No. 2M is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1M.

Sequence ID No. 2N is the amino acid sequence of  
25 an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1N.

204070-268300F

Sequence ID No. 2P is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 1P.

Sequence ID No. 3 is a nucleotide sequence  
5 encoding the 63 nucleotide insert present in Sequence ID Nos. 1C, 1G, 1H, 1I, 1M, 1N and 1P.

Sequence ID No. 4 is the 21 amino acid sequence encoded by the insert set forth in Sequence ID No. 3.

Sequence ID No. 5 is a nucleotide sequence of a  
10 clone (pCMV-26-NotI-24) encoding a human N-methyl-D-aspartate (NMDA) receptor subunit, NMDAR2C, and the deduced amino acid sequence thereof.

Sequence ID No. 5A is a 2026 nucleotide sequence encoded by clone NMDA21, comprising nucleotides 931 - 2350,  
15 and 2402 - 3307 of Sequence ID No. 5. Thus, Sequence ID No. 5A differs from Sequence ID No. 5 in that it does not contain the 930 5' nucleotides thereof, nor the 51 nucleotides located at position 2351 - 2401 of Sequence ID No. 5, nor the 1061 3' nucleotides of Sequence ID No. 5.

Sequence ID No. 5B is a 3698 nucleotide sequence encoded by clone NMDA22, comprising nucleotides 367 - 1300  
20 of Sequence ID No. 5, plus an additional 11 nucleotides (set forth as Sequence ID No. 9), and nucleotides 1301 - 1959 and 1975 - 4068 of Sequence ID No. 5. Thus, Sequence  
25 ID No. 5B differs from Sequence ID No. 5 by the lack of the 366 5'-most nucleotides, by the insertion of 11 nucleotides between nucleotides 1300 and 1301 of Sequence ID No. 5, and further by the lack of the 15 nucleotides of Sequence ID No. 5 from residue 1960 to residue 1974.

204070-258E00T

Sequence ID No. 5C is a 3243 nucleotide sequence encoded by clone NMDA24, comprising nucleotides 861 - 1300 of Sequence ID No. 5, plus an additional 11 nucleotides (Sequence ID No. 9), nucleotides 1301 - 2350 of Sequence ID  
5 No. 5, an additional 24 nucleotides (set forth as Sequence ID No. 7) and nucleotides 2351 - 4068 of Sequence ID No. 5. Thus, Sequence ID No. 5C differs from Sequence ID No. 5 in that it does not contain the 860 5'-most nucleotides thereof, while it does contain an additional 11 nucleotides  
10 (Sequence ID No. 9) inserted between nucleotides 1300 and 1301, plus an additional 24 nucleotides (Sequence ID No. 7) inserted between nucleotides 2350 and 2351 of Sequence ID No. 5.

Sequence ID No. 5D is a 3025 nucleotide sequence  
15 encoded by clone NMDA26, comprising nucleotides 1 - 3025 of Sequence ID No. 5. Thus, Sequence ID No. 5D differs from Sequence ID No. 5 in that it does not contain the 1043 3'-terminal nucleotides thereof.

Sequence ID No. 5E is a nucleotide sequence  
20 encoding human NMDA receptor subunit pCMV-26-ScaI-24, which differs from Sequence ID No. 5 only in the insertion of 24 nucleotides (Sequence ID No. 7) between nucleotides 2350 and 2351 of Sequence ID No. 5.

Sequence ID No. 5F is a nucleotide sequence  
25 encoding human NMDA receptor subunit pCMV-26-ScaI-22, which differs from Sequence ID No. 5 only in the deletion of nucleotides 1960 - 1974 of Sequence ID No. 5.

Sequence ID No. 5G is a nucleotide sequence encoding human NMDA receptor subunit pCMV-26-ScaI-21-NotI-24,  
30 which differs from Sequence ID No. 5 only in the deletion of nucleotides 2351 - 2401 of Sequence ID No. 5.

204070-2688001

Sequence ID No. 5H is a nucleotide sequence encoding human NMDA receptor subunit NMDAR2C- $\Delta$ 15-I24. Sequence ID No. 5H is the same as Sequence ID No. 5F, except Sequence ID No. 5H further contains the 24  
5 nucleotide insert set forth in Sequence ID No. 7, positioned between nucleotides 2350 and 2351 of Sequence ID No. 5.

Sequence ID No. 5I is a nucleotide sequence encoding human NMDA receptor subunit NMDAR2C- $\Delta$ 15- $\Delta$ 51.  
10 Sequence ID No. 5I is the same as Sequence ID No. 5G, except Sequence ID No. 5I does not contain the 15 nucleotides set forth as nucleotides 1960 - 1974 of Sequence ID No. 5.

Sequence ID No. 6 is the amino acid sequence of  
15 the NMDA receptor subunit set forth in Sequence ID No. 5.

Sequence ID No. 6A is the amino acid sequence of a portion of an NMDA receptor subunit as encoded by the nucleotide sequence of Sequence ID No. 5A.

Sequence ID No. 6B is the amino acid sequence of  
20 a portion of an NMDA receptor subunit as encoded by the nucleotide sequence of Sequence ID No. 5B.

Sequence ID No. 6C is the amino acid sequence of a portion of an NMDA receptor subunit as encoded by the nucleotide sequence of Sequence ID No. 5C.

Sequence ID No. 6D is the amino acid sequence of  
25 a portion of an NMDA receptor subunit as encoded by the nucleotide sequence of Sequence ID No. 5D.

Sequence ID No. 6E is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence  
30 of Sequence ID No. 5E.

204070-010402



Sequence ID No. 6F is the amino acid sequence of an NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 5F.

Sequence ID No. 6G is the amino acid sequence of receptor subunit encoded by the nucleotide sequence of Sequence ID No. 5G.

Sequence ID No. 6H is the amino acid sequence of receptor subunit encoded by the nucleotide sequence of Sequence ID No. 5H.

Sequence ID No. 6I is the amino acid sequence of NMDA receptor subunit encoded by the nucleotide sequence of Sequence ID No. 5I.

Sequence ID No. 7 is a nucleotide sequence encoding the 24 nucleotide insert present in Sequence ID Nos. 5C, 5E and 5H.

Sequence ID No. 8 is the 7 amino acid sequence encoded by nucleotides 2-22 of the insert set forth in Sequence ID No. 7. Because the insert is introduced within a codon, the insert itself only encodes 7 amino acids. The 20 terminal residues of the nucleotide insert participate in forming codons with adjacent sequence at the site of insertion.

Sequence ID No. 9 is a nucleotide sequence encoding the 11 nucleotide insert present in Sequence ID 25 Nos. 5B and 5C.

Sequence ID No. 10 is a nucleotide sequence encoding a human N-methyl-D-aspartate (NMDA) receptor subunit, NMDAR2A.

no acid sequence of  
by the nucleotide

side sequence of  
clone NMDA27,  
- 74) of said

quence of a  
) receptor

ence of  
No. 13.

of a  
ctor

f

Sequence ID No. 11 is the amino acid sequence of an NMDA receptor subunit as encoded by the nucleotide sequence set forth in Sequence ID No. 10.

Sequence ID No. 12 is the nucleotide sequence of 5 71 nucleotides of 5' untranslated sequence of clone NMDA27, plus the initiation codon (nucleotides 72 - 74) of said clone.

Sequence ID No. 13 is a nucleotide sequence of a clone encoding a human N-methyl-D-aspartate (NMDA) receptor 10 subunit, NMDAR2B.

Sequence ID No. 14 is the amino acid sequence of the NMDA receptor subunit set forth in Sequence ID No. 13.

Sequence ID No. 15 is a nucleotide sequence of a clone encoding a human N-methyl-D-aspartate (NMDA) receptor 15 subunit, NMDAR2D.

Sequence ID No. 16 is the amino acid sequence of the NMDA receptor subunit set forth in Sequence ID No. 15.

Sequence ID Nos. 17-20 are four synthetic oligonucleotides used in the preparation of an NMDAR2C 20 clone (pCMV-26-NotI-24-GCMOD) having reduced GC nucleotide content between nucleotides 2957 and 3166.

Sequence ID No. 21 is the nucleotide sequence of the 195 basepair insert of NMDAR2C clone pCMV-26-NotI-24-GCMOD (replacing nucleotides 2966-3160 of 25 Sequence ID No. 5).